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Original Research

Analysis of circulating tumour DNA to identify patients with epidermal growth factor receptor–positive non-small cell lung cancer who might benefit from sequential tyrosine kinase inhibitor treatment

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Abstract ***Background:*** Survival data support the use of first-line osimertinib as the standard of care for epidermal growth factor receptor (*EGFR*)–positive non-small cell lung cancer (NSCLC). However, it remains unclear whether upfront osimertinib is superior to sequential first- or second-generation tyrosine kinase inhibitors (TKIs) followed by osimertinib for all patients. It is impossible to predict which patients are at high risk of progression, and this constitutes a major limitation of the sequential TKI approach.

Patients and methods: A total of 830 plasma samples from 228 patients with stage IV, *EGFR*-positive NSCLC who were treated with first-line TKIs were analysed by digital polymerase chain reaction (dPCR).

Results: The circulating tumour DNA (ctDNA) levels helped to identify patients with significantly improved survival rate, regardless of the treatment. Patients treated with first- or second-generation TKIs (N = 189) with *EGFR* mutations in plasma at a mutant allele frequency (MAF) <7% before treatment initiation (low-risk patients) or who were ctDNA negative after 3 or 6 months of treatment and with an MAF <7% at diagnosis (high responders) had two-thirds lower risk of death than patients in the opposite situation (adjusted hazard ratio [HR] = 0.38; 95% confidence interval [CI]: 0.23–0.64 and HR = 0.22; 95% CI: 0.12–0.42, respectively). The median overall survival (OS) for low-risk patients and high responders treated with first- or second-generation TKIs was 34.2 months and not reached, respectively, regardless of second-line treatment. There were no significant difference in OS between low-risk or high-responder patients treated upfront with osimertinib (N = 39) and those treated under a sequential approach with osimertinib (N = 60). Median OS was not reached in both cases.

Conclusions: Pre-treatment ctDNA levels identify low-risk patients, who may benefit from sequential TKI treatment. Information regarding *EGFR* mutation clearance can help to improve patient selection.

1. Introduction

Lung cancer remains a deadly disease and a major health problem, with the 5-year survival rate being less than 25% in patients with non–small cell lung cancer (NSCLC) [1]. Epidermal growth factor receptor (*EGFR*)–activating mutations confer tumour sensitivity to tyrosine kinase inhibitors (TKIs), which have significantly improved the survival and quality of life for patients with NSCLC [2–5].

Osimertinib is a third-generation TKI that has demonstrated clinical activity as a second-line treatment in patients with *EGFR*-positive NSCLC harbouring the T790M resistance mutation [6]. In the FLAURA trial, osimertinib showed improved progression-free survival (PFS) and overall survival (OS) compared with those of first-generation TKIs in first-line treatment, changing the treatment paradigm of *EGFR*-positive NSCLC [7,8]. However, it is still unclear whether upfront osimertinib is superior to sequential treatment with first- or second-generation TKIs, followed by osimertinib in all cases. In accordance with recent data, a median OS of 41.3 months was achieved in patients with *EGFR* T790M–positive NSCLC who were treated with sequential afatinib plus osimertinib [9]. The identification of patients with *EGFR*-positive NSCLC at high risk of progression is crucial to achieve optimal management of the disease and maximise the survival rate. In recent years, liquid biopsy has emerged as an attractive tool for treatment monitoring. Circulating tumour DNA (ctDNA) has emerged as a blood sample–based, non-invasive biomarker in testing and monitoring the

tumour response to treatment within a narrow time frame [10,11].

We present a prospective cross-sectional study conducted by the Spanish Lung Cancer Group (SLCG) that was specifically designed to evaluate the prognostic value of ctDNA levels in 830 plasma samples from 228 patients with *EGFR*-positive NSCLC.

2. Materials and methods

2.1. Study cohort

A prospective, multicentre, observational study was conducted by the SLCG (https://www.gecp.org/). The study was approved by the Ethical Committee of Hospital Puerta de Hierro, Madrid, Spain (internal code: PI 02/16), and had been conducted in accordance with the precepts of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

All patients had to sign an informed consent to be included in the study. Eligible patients were either men or women, were ≥18 years old, had pathologically confirmed stage IV tumour (as per the criteria of the American Joint Committee on Cancer, 7th edition, criteria), had *EGFR*-mutated NSCLC and were candidates for first-line TKI treatment. The TKI type was selected according to the investigator's criteria. Life expectancy had to be over 12 weeks.

Two hundred seventy-five patients were initially recruited from 28 centres between February 2016 and September 2020. Forty-one patients were excluded as they did not meet the inclusion criteria or because data for subsequent analyses were not available. Patients with exon 20 insertion tumour or more than one driver mutation in the *EGFR* gene were also excluded from the analyses (N = 6). Finally, a total number of 228 patients from 25 centres were included in the study (Fig. 1).

A complete workup was performed before recruiting the patients for the study. Data on demographic characteristics, clinicopathological features, *EGFR* mutation, vital status, disease status, treatment, toxicity, drug dose adjustments or discontinuation of medication were collected. Electronic data collection was performed using a specific web-based platform developed by Xolomon Tree S.L. The patients' information was kept hidden from the laboratory. Computed tomography measurements and magnetic resonance imaging were performed at 3 and 6 months after TKI treatment initiation. Treatment response was assessed as per Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 criteria.

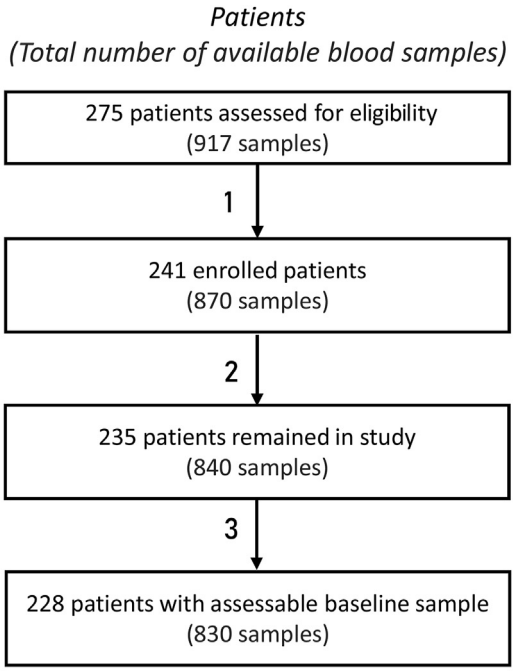
2.2. Laboratory procedures

Four samples were taken from each of the patients: (1) at stage IV NSCLC diagnosis, before initiation of first-line treatment with a TKI, (2) at 3-month re-evaluation after TKI initiation, (3) at 6-month re-evaluation after TKI initiation and, finally, (4) at the time of disease progression (Supplementary Fig. 1). Only a variation of ±1 month was permitted for each sample. Additional samples taken at other intermediate points were also accepted. Initially, a total of 917 blood samples were collected. Samples from patients who did not meet the inclusion criteria or those who were not suitable for molecular analyses were excluded. The final number of samples analysed was 830 (Fig. 1).

Blood samples were collected in 8.5-mL PPT™ tubes (Becton Dickinson). The samples were first centrifuged at 1600 *g* for 10 min at room temperature. Subsequently, a second round of centrifugation was performed at 6000 *g* for 10 min. Haemolysed samples were excluded from further molecular analysis. Circulating free DNA (cfDNA) was extracted from plasma using the cfDNA QIAmp Circulating Nucleic Acid kit (Qiagen®) following the manufacturer's recommendations. The cfDNA was stored at −80°C until further analysis. *EGFR* mutations were analysed by using digital polymerase chain reaction (dPCR). The presence of mutations in cfDNA was evaluated using predesigned TaqMan® dPCR assays in a QuantStudio® 3D Digital PCR System (Applied Biosystems, South San Francisco, CA, United States of America). dPCR reactions were performed in a final volume of 18 μL using 8.55 μL of cfDNA as a template. Subsequently, 14.5 μL of the reaction was loaded into a QuantStudio 3D Digital PCR 20K chip. Cycling conditions were as follows: initial denaturation at 96 C for 10 min and 40 cycles at 56 C for 2 min, 98 C for 30 s and 72 C for 10 min, and finally, the samples were maintained at 22 C for at least 30 min. Chip fluorescence was read twice using two independent detectors. The results were analysed using QuantStudio® 3D AnalysisSuite™ Cloud Software. Automatic call assignments for each data cluster were manually adjusted when needed. Positive and negative controls were included in every run. Mutant allele frequency (MAF) was defined as the number of mutant molecules at a specific nucleotide location relative to the sum of mutant and wild-type DNA molecules. The detection limit for each assay had been published in the study by Provencio et al [10]. The sensitivity and specificity of the assays, considering tissue genotyping to be the gold standard, have also been reported [12].

2.3. Statistical analyses

The primary objective of the study was to evaluate the prognostic value of ctDNA quantification at specific time points. The secondary objectives included evaluation of associations between MAF fluctuations (difference in MAF obtained between the first and second



Filter	Number of excluded patients	Reason
1	34	Patients who do not keep to inclusion criteria: • Time of survival less than 12 weeks (n=18) • No TKI treatment (n=8) • KRAS mutation detection at diagnosis (n=2) • Early lost to follow-up (n=6)
2	6	Patients whose tumours harbour ins20 mutations or more than 1 mutation in EGFR
3	7	Patients whit baseline blood sample non-assessable

Fig. 1. **Study scheme**. Description of patients and samples finally included in the study cohort. Forty-one patients were excluded as they did not meet the inclusion criteria or because data for subsequent analyses were not available. Patients whose tumours harboured an insertion in exon 20 or with more than one driver mutation in the EGFR gene were also excluded from the analysis (N = 6). Initially, a total of 917 blood samples were collected. Samples from patients who did not meet the inclusion criteria or who were not suitable for molecular analysis were excluded. The final number of samples analysed was 830. EGFR, epidermal growth factor receptor.

samples or between the first and third samples), PFS and OS.

Categorical variables are summarised as frequencies, whereas continuous variables are displayed as the mean and standard deviation; non-normally distributed variables are displayed as the median and 25th and 75th percentiles. Categorical variables were compared using the chi-square test, Fisher's exact test or analysis of variance, whichever was the most appropriate. Continuous variables were compared using Student's t-test or the Mann-Whitney U test.

Median follow-up was estimated using the reverse Kaplan-Meier method. OS and PFS were evaluated using the Kaplan-Meier survival function and Cox proportional hazards models. For OS analysis, time from the start of treatment with a TKI to death or last follow-up was obtained. PFS was defined as the time between the start of first-line TKI treatment and disease progression, as assessed by RECIST criteria, the censored date of the last follow-up or exit, whichever occurred first. The proportional hazards assumption underlying the Cox model was checked based on scaled Schoenfeld residuals. Hazard ratios (HRs) and the corresponding 95% confidence intervals (95% CIs) were estimated from the Cox model using a multivariate approach adjusted for potential confounding factors. In the adjusted model, sex, Eastern Cooperative Oncology Group (ECOG) performance status, histology and the number of extrathoracic metastases were included.

The statistical significance level was set at 0.05. Statistical analyses were carried out using Stata version 16.0 (StataCorp 2019, Stata Statistical Software Release 16; StataCorp LLC, College Station, TX).

3. Results

3.1. Patient characteristics

Demographic and clinical characteristics of all the 228 patients included in the analyses are shown in Table 1.

Table 1
Clinicopathological characteristics of the study population.

Clinicopathological characteristics	N = 228
Age, mean (SD), years	67.6 (11.7)
Sex,[a] no. (%) with data	
Female	135 (59.2)
Male	91 (39.9)
NA	2 (0.9)
Smoking,[a] no. (%) with data	
Never-smokers	129 (56.6)
Former smokers	74 (32.4)
Active smokers	23 (10.1)
NA	2 (0.9)
Cigarettes per day (median; P25; P75)	
Former smoker	10 (4; 20)
Active smokers	19.5 (10; 20)
Performance status,[b] no. (%) with data	
0	75 (32.9)
1	93 (40.8)
2	16 (7)
3	5 (2.2)
NA	39 (17.1)
Stage, no. (%) with data	
IVA	108 (47.4)
IVB	120 (52.6)
Histology, no. (%) with data	
Adenocarcinoma	210 (92.1)
Adenosquamous	7 (3.1)
Large cell	7 (3.1)
Squamous	4 (1.7)
Previous cancer,[c] no. (%) with data	
Yes	20 (8.8)
No	168 (73.7)
NA	40 (17.5)
Metastases,[d] no. (%) with data	
Local	134 (58.8)
Bone	91 (39.9)
CNS	40 (17.5)
Liver	29 (12.7)
Other	5 (2.2)
Sum of extrathoracic metastases,[d] no. (%) with data	
None	58 (25.4)
One	86 (37.7)
Two	35 (15.3)
Three	16 (7)
EGFR mutation, no. (%) with data	
Del19	137 (60.1)
G719X	6 (2.6)
L858R	79 (34.7)
L861Q	6 (2.6)
First-line treatment, no. (%) with data	
Afatinib	84 (36.8)
Erlotinib	45 (19.7)
Gefitinib	60 (26.3)
Osimertinib	39 (17.1)
Second-line treatment,[e] no. (%) with data	
TKIs	2 (1.2)
Chemotherapy	26 (15.8)
Osimertinib	60 (36.4)
Palliative care	5 (3)
Immunotherapy	1 (0.6)
None	31 (18.8)
NA	40 (24.2)

SD, standard deviation; TKI, tyrosine kinase inhibitor; EGFR, epidermal growth factor receptor; CNS, central nervous system; P, percentiles.
[a] Two patients without information.
[b] Thirty-nine patients without information.
[c] Forty patients without information.
[d] Thirty-three patients without information.
[e] Thirty-one without second-line treatment and 40 patients without information.

Patients were predominantly Caucasian (97%); 59.2% were women; the diagnosis age ranged from 26 to 90 years, with a mean of 68 years and 56.6% were never-smokers. In accordance with clinical reports, 92.1% of the cases were adenocarcinomas, and the majority (52.6%) were stage IVB with either 1 or more extra-thoracic metastases (60%). In total, 189 patients (82.9%) received first-line treatment with afatinib, gefitinib or erlotinib. Of these, 60 (31.7%) received osimertinib as a second-line treatment. In addition, 39 (17.1%) patients were treated with first-line osimertinib.

EGFR mutation status in tissue biopsy was assessed locally. According to pathologists' reports, 94.8% of patients harboured one of the most common *EGFR*-activating mutations: exon 19 deletion (60.1%) or exon 21 point mutation L858R (34.7%). Other uncommon *EGFR* mutations were also found. There were no significant differences in terms of demographic and clinical characteristics between patients with common *EGFR-activating mutations* and those with uncommon *EGFR*-activating mutations (Supplementary Table 1). The original *EGFR*-sensitising mutation was detected in 78.1% of the pre-treatment samples.

3.2. Clinical outcomes

The median follow-up was 28.0 months (95% CI: 26.6–30.3). During the study, 99 deaths were registered, and 165 patients progressed to treatment with a first-line TKI.

In the univariate analyses, sex, ECOG performance status, histology, smoking status, mutation type (common vs. uncommon) and the total number of extra-thoracic metastases were associated with OS and PFS ($P < 0.05$ in all cases; Supplementary Figs. 2 and 3). Patients treated with first-line osimertinib did not attain median PFS (95% CI: 13.3-not reached [NR]; N = 39), whereas patients treated with a first- or second-generation TKI achieved a median PFS of 11.8 months (95% CI: 10.6–12.9; N = 189) (HR = 0.29, 95% CI: 0.15–0.56). There were no significant differences in survival based on first- or second-generation TKIs (afatinib, erlotinib or gefitinib) (Supplementary Fig. 4).

The median OS was 22.8 months (95% CI: 16.1–NR) for patients treated with first- or second-generation TKIs, followed by second-line treatment other than osimertinib (N = 129); 32 months (95% CI: 23.9–NR) for patients receiving a first-line gefitinib, erlotinib or afatinib, followed by a second-line treatment with osimertinib (N = 60) and NR for patients treated with first-line osimertinib (N = 39) (95% CI: 17.0–NR) (Supplementary Fig. 5) (P = 0.028).

Baseline characteristics based on the treatment strategy are presented in Supplementary Table 2. As presented, there were no significant differences in the distribution of clinicopathological features among patients treated with upfront osimertinib (N = 39), those

treated with sequential first- or second-generation TKIs, followed by osimertinib (N = 60) or those who never received osimertinib (N = 129), except for the number of extrathoracic metastases.

Regarding toxicity, adverse events were reported in 101 patients. The most frequent toxicities were diarrhoea (23.7%) and rash (26.8%) (Supplementary Table 3).

3.3. ctDNA levels predict survival in patients treated with first-line osimertinib

In total, 39 patients were treated with first-line osimertinib. The prognostic values of different MAF thresholds (≥5%, ≥7% and ≥10%) measured in the pre-treatment plasma sample were evaluated. The results were similar with all cut-off points, and 7% MAF was selected (Supplementary Table 4). Patients with ctDNA detected at an MAF ≥7% in the pre-treatment sample had significantly worse PFS, with a median PFS of 12.6 months (95% CI: 11.9–NR) compared with NR (95% CI: 26.1–NR) in low-risk patients (defined as patients with *EGFR*-sensitising mutations, at baseline, detected at an MAF <7%). Likewise, the median OS was 17.0 months (95% CI: 13.4–NR) in patients with high ctDNA levels before treatment (MAF ≥7%) and NR (14.7–NR) for low-risk patients.

3.4. ctDNA levels predict survival in patients treated with first-line, first- or second-generation TKIs

Overall, 189 patients were treated with a first-line afatinib, erlotinib or gefitinib. Again, low-risk patients had significantly improved PFS and OS compared with patients in the opposite situation (adjusted HR = 0.51; 95% CI: 0.34–0.78 and HR = 0.38; 95% CI: 0.23–0.64, respectively) (Table 2 and Fig. 2). Likewise, patients who became plasma ctDNA negative after 3 months of treatment exhibited improved PFS (adjusted HR = 0.45; 95 CI: 0.26–0.78) and OS (HR = 0.35; 95% CI: 0.19–0.65). Similarly, undetectable levels of ctDNA after 6 months of treatment were associated with significantly longer PFS and OS (HR = 0.42; 95% CI: 0.27–0.65 and HR = 0.29; 95% CI: 0.15–0.56, respectively) (Table 2).

When classifying these patients as 'responders' to first- or second-generation TKIs (those who achieved negative status after 3 or 6 months of treatment) and 'non-responders' (those who did not), analogous results were obtained in the multivariate analyses (data not shown). When the subset of patients with ctDNA levels with MAF ≥7% in the pre-treatment sample (N = 31) within the responder group is compared with those patients with lower ctDNA levels (<7% MAF) (N = 103), the latter from now on designated as high responders; a significantly improved prognosis in terms of PFS and OS was found in high responders (adjusted HR = 0.30; 95% CI: 0.19–0.49 and HR = 0.22; 95% CI: 0.12–0.42, respectively) (Fig. 2 and Table 2). The median PFS for high responders was 15 months (95% CI: 11.9–17.8), and the median OS was NR (95% CI: 32.6–NR), whereas for responders with ≥7% MAF, the median PFS and OS were 12.2 (95% CI: 9.5–16.4) and 24.1 (95% CI: 18.5–31.6) months, respectively. Both groups also had consistently better PFS and OS than the non-responder

Table 2
Median PFS and OS, hazard ratios and the corresponding 95% CI in patients treated with first-line first- or second-generation TKIs and based on ctDNA levels at baseline, 3-month follow-up and 6-month follow-up.

Group of patients based on ctDNA levels measured as MAFs		Median (95% CI), months	Hazard ratio (95% CI)[a]	P value[a]
PFS	Cut-off ≥7% MAF at baseline	8.6 (6.5–10.6)	1.0 (reference category)	0.002
	Cut-off <7% MAF at baseline	12.6 (11.7–15.8)	0.51 (0.34–0.78)	
	Cut-off ≥0.1% MAF at 3 months	5.9 (4.8–8.6)	1.0 (reference category)	0.004
	Cut-off <0.1% MAF at 3 months	12.4 (11.6–16.4)	0.45 (0.26–0.78)	
	Cut-off ≥0.1% MAF at 6 months	10.5 (8.6–12.2)	1.0 (reference category)	<0.001
	Cut-off <0.1% MAF at 6 months	17.1 (15.0–21.1)	0.42 (0.27–0.65)	
OS	Cut-off ≥7% MAF at baseline	17.5 (11.3–23.2)	1.0 (reference category)	<0.001
	Cut-off <7% MAF at baseline	34.2 (30.9–NR)	0.38 (0.23–0.64)	
	Cut-off ≥0.1% MAF at 3 months	12.8 (6.7–22.8)	1.0 (reference category)	0.001
	Cut-off <0.1% MAF at 3 months	33 (26.8–NR)	0.35 (0.19–0.65)	
	Cut-off ≥0.1% MAF at 6 months	22.8 (16.1–26.8)	1.0 (reference category)	<0.001
	Cut-off <0.1% MAF at 6 months	NR (33–NR)	0.29 (0.15–0.56)	
PFS	Non-responders	6.7 (5.3–10.8)	1.0 (reference category)	–
	Responders with ≥7% MAF at baseline	12.2 (11.9–17.8)	0.47 (0.26–0.83)	0.010
	Responders with <7% MAF at baseline	15.0 (11.9–17.8)	0.30 (0.19–0.49)	<0.001
OS	Non-responders	14.1 (9.4–22.8)	1.0 (reference category)	–
	Responders with ≥7% MAF at baseline	24.1 (18.5–31.6)	0.61 (0.32–1.18)	0.144
	Responders with <7% MAF at baseline	NR (32.6-NR)	0.22 (0.12–0.42)	<0.001

Median PFS and OS, hazard ratios and the corresponding 95% CI in non-responders, high responders (responders with ctDNA at <7% MAF at baseline) and responders with ctDNA at ≥7% MAF at baseline.

CI, confidence interval; MAF, mutant allele frequency; PFS, progression-free survival; OS, overall survival; ctDNA, circulating tumour DNA; NR, not reached; TKI, tyrosine kinase inhibitor.

[a] Data adjusted by multivariate analysis.

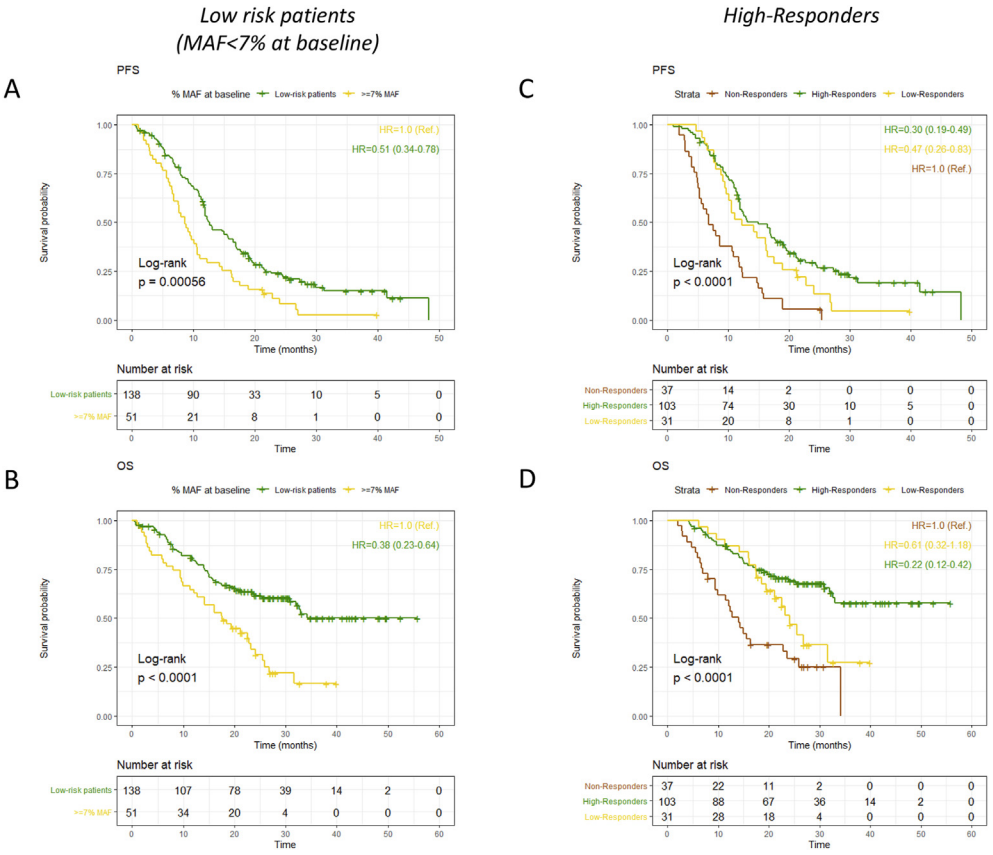


Fig. 2. **Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) for low-risk patients and high responders**. Kaplan-Meier curves for OS and PFS based on mutant allele frequency (MAF) measured in the pre-treatment plasma sample (A and B). Survival curves for patients with an MAF <7% at baseline are coloured in green (A and B). PFS and OS curves based on MAF measured in the pre-treatment plasma sample and after 3 and 6 months (C and D). Curves for high responders (patients with an MAF <7% at baseline and undetectable ctDNA at 3 or 6 months) are coloured in green. Hazard ratio (95% CI) and log-rank test P-values are shown in each graph. ctDNA, circulating tumour DNA; HR, hazard ratio; CI, confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

group (N = 37). It is important to point out that among these patients (N = 189), only 60 of them were treated with osimertinib after the first disease progression.

3.5. Low-risk patients and high responders might benefit from sequential treatment

As an exploratory analysis, we examined whether low-risk patients (defined as those with ctDNA status at an MAF <7% in the pre-treatment sample) and high responders (those who achieved negative status after 3 or 6 months of treatment and with baseline ctDNA levels at <7% MAF) could potentially benefit from sequential treatment with afatinib, erlotinib or gefitinib, followed by osimertinib. We compared OS between patients who received osimertinib on progression to a first- or second-generation TKI (N = 60) and those treated upfront with osimertinib (N = 39).

Table 3
Median OS, hazard ratios and the corresponding 95% CI for patients treated with upfront osimertinib and low-risk/high-responder patients treated with first-line treatment with afatinib, gefitinib or erlotinib followed by osimertinib.

Group of patients based on when they have received osimertinib		Median (95% CI), months	Hazard ratio (95% CI)[a]	P value[a]
Low-risk	Upfront osimertinib	NR (14.7–NR)	1.0 (reference category)	–
	Second-line osimertinib	NR (32–NR)	1.13 (0.82–1.55)	0.454
High responders	Upfront osimertinib	NR (28.6–NR)	1.0 (reference category)	–
	Second-line osimertinib	NR (32–NR)	1.32 (0.78–2.22)	0.297

OS, overall survival; NR, not reached; CI, confidence interval.
[a] Data unadjusted.

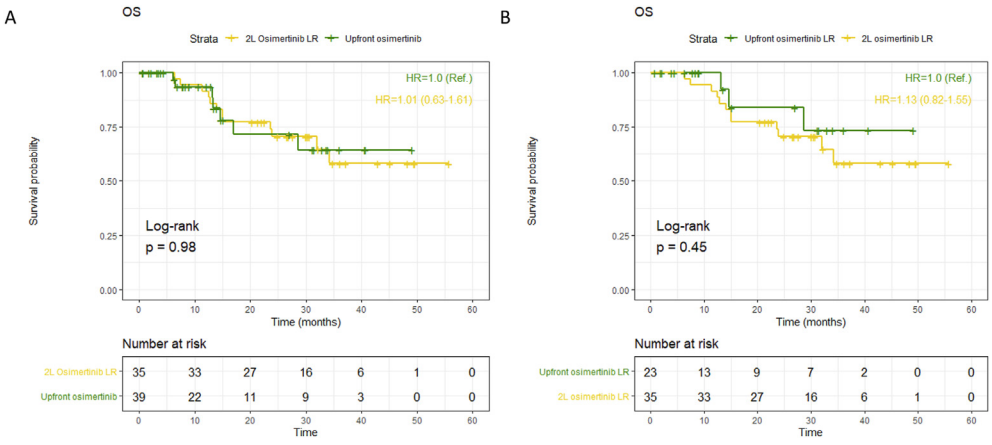


Fig. 3. **Kaplan-Meier curves for overall survival for patients treated with upfront osimertinib and low-risk patients treated with first-line treatment afatinib, gefitinib or erlotinib followed by osimertinib (A). Kaplan-Meier curves for overall survival for low-risk patients treated with upfront osimertinib and sequential approach (B)**. Hazard ratio (95% CI) and log-rank test P-values are shown in each graph. CI, confidence interval; HR, hazard ratio.

In accordance with our results, the median OS was NR in low-risk patients and high responders to sequential treatment, with a 95% CI of 32.0–NR in both cases (the median follow-up was 30.5 months for these patients) (Table 3). Similar results were observed for the equivalent subset of patients treated with first-line osimertinib (median OS = NR in both cases; 95% CI: 14.7–NR for low-risk patients and 28.6–NR for high responders). However, the median follow-up for this subset of patients was lower. In the same way, Kaplan-Meier plots presented in Fig. 3 show that similar survival curves were obtained for low-risk patients treated either with upfront osimertinib or a sequential strategy.

3.6. T790M status at disease progression in patients treated with first- or second-generation TKIs

The presence of the T790M resistance mutation in ctDNA was evaluated in all the samples. The resistant T790M mutation was always detected along with the original *EGFR*-sensitising mutation. The T790M mutation was detected only in one of the pre-treatment samples. Among patients who progressed on first-line afatinib, gefitinib or erlotinib and in whom the resistance mutation was detected after 3 or 6 months of TKI treatment (N = 28), 25 experienced disease progression during the study. The remaining 3 cases had stable disease. In line with expectations, plasma detection of the T790M mutation after 6 months of treatment was associated with a high risk of disease progression and death (adjusted HR = 2.61; 95% CI: 1.43–4.73 and HR = 2.14; 95% CI: 1.06–4.33, respectively).

On disease progression, the T790M mutation was detected in 52.8% of patients. The T790M mutation was more frequently detected in patients in whom ctDNA was detectable before treatment initiation compared with those in whom it was non-detectable (P = <0.001). In fact, there was a significant association between *EGFR*-sensitising MAF before treatment initiation and T790M detection in plasma at disease progression (Supplementary Fig. 7).

The detection probability of T790M resistance mutation in blood was also higher among never-smokers (P = 0.010) and patients with bone and liver metastases (P = 0.032 and P = 0.002, respectively).

4. Discussion

Our study demonstrates that ctDNA levels can be a factor of better or worse prognosis in patients with *EGFR*-positive NSCLC treated with first- or second-generation TKIs and independent of the influence of other well-established prognostic factors.

Since the publication of the results of the FLAURA trial, which clearly demonstrated superior median PFS and OS in patients treated with osimertinib (18.9 and 38.6 months, respectively) versus those treated with first-generation TKIs (10.2 and 31.8 months, respectively) [7,8], an intense debate is ongoing between those who propose an upfront use of osimertinib and those who advocate for a sequential approach using first- or second-generation TKIs and relegating osimertinib to rescue patients with disease progression [9].

An important limitation of the sequential approach is that it is impossible to identify a group of patients with a better prognosis, beyond the observations of the presence of exon 19 deletion [13], a significant prognostic factor that is neither easily explained in pathophysiological terms nor universally accepted. In our study, no significant differences in survival with respect to the type of common mutation were found.

In accordance with our results, patients treated under a sequential approach (N = 189) with an MAF <7%

had a median OS of 34.2 months (95% CI: 30.9–NR), very similar to the OS of 38.6 months (95% CI: 34.5–41.8) that was reported in the FLAURA study. Remarkably, OS in our series was achieved by treating more than half of the patients with a drug other than osimertinib, after progression to a TKI. By contrast, detecting an *EGFR*-sensitising mutation with an MAF ≥7% before treatment initiation identifies a group of patients with significantly worse outcomes.

In our opinion, the effect size was clinically relevant as patients with an *EGFR*-sensitising mutation at an MAF <7% before treatment initiation had a 96% increase in duration of PFS compared with that of patients with an MAF ≥7% (adjusted HR = 0.51; 95% CI: 0.34–0.78 for PFS). In the same manner, the adjusted HR for OS was 0.38 (95% CI: 0.23–0.64).

Restricting the analyses to patients treated with first- or second-generation TKIs followed by osimertinib (N = 60), median OS was unattainable in low-risk patients or high responders, with a 95% CI of 32.0–NR in both cases (the median follow-up was 30.5 months for these patients). In the FLAURA trial, the median OS time was 38.6 months. Our data are not mature enough to compare the two data sets, but the lower confidence limit in this subset of patients (32 months) is close to that reported in the FLAURA trial (34.5 months). In addition, in accordance with our data, similar results were obtained for the equivalent subset of patients treated with first-line osimertinib. However, the median follow-up for this subset of patients is lower, which may constitute an important limitation.

Our results can be translated into daily clinical practice and have several strong points in their favour. First, this was a prospective, multicentre study in which samples were collected and analysed based on a strict protocol. For the sequencing approach, the median PFS in our series was 11.8 months (95% CI: 10.0–14.7) and the median OS was 32.0 months (95% CI: 23.9–NR). These findings concur with others in the literature [14] and the meta-analysis of six randomised trials in treatment-naive patients with *EGFR* mutation who achieved a median PFS of 11 months compared with 5.6 months of chemotherapy [15], demonstrating the lack of any significant selection bias. The *EGFR*-sensitising mutation was detected in 78.1% of pre-treatment samples, which is again in line with the literature [16,17]. The association between tumour burden and incidence of bone or liver metastases has been published by other investigators, both in TKI-treated and chemotherapy-treated patients [18].

An additional important differential factor in our study is ctDNA quantification. Numerous studies evaluating the prognostic value of ctDNA detection have been conducted, but few have specifically included quantification in prospectively collected samples at specific time points [19]. A recent retrospective study in multiple tumour types, including 16 lung cancers, found an association of high MAF levels with worse OS [20]. Another study on 37 patients with metastatic colon cancer also yielded similar findings [21].

An important question that remains unanswered is whether sequential quantification of ctDNA is useful for the monitoring of patients with *EGFR* mutation. Traditionally, reduction in tumour size during treatment has been associated with a better prognosis. However, in the case of patients with *EGFR* mutations, data on the relationship between clinical responses and survival are controversial. An analysis of individual data from the EURTAC, IPASS, ENSURE, LUX-Lung 3 and LUX-Lung 6 trials did not find differences between the grades of response at 6 or 12 weeks with PFS or OS. Therefore, there are no clear predictive surrogate markers of survival that can be useful for the management of these patients [22]. In our cohort, *EGFR*-sensitising MAFs measured after 3 and 6 months of treatment were also of prognostic significance. As presented, survival was significantly better for those patients classified as responders (with undetectable ctDNA at 3 or 6 months). These results agree with other studies including fewer patients such as in the study by Mok *et al.* [23] or a published series on Caucasian patients wherein the clearance of ctDNA was analysed using the Cobas® method [24]. Recent data on breast cancer also indicate that the temporal dynamics of ctDNA may even yield more information than baseline levels of a given mutation at diagnosis [25]. Similarly, several small studies on Asian patients have demonstrated that negative ctDNA status after 4 weeks of treatment—without differences observed in tumour size as per RECIST criteria—predicts longer PFS compared with patients who do not become ctDNA negative [16,26]. This is probably because MAF yields a complete picture of disease spreading and aggressivity that complements conventional analytic or radiographic tests [27]. Similar results were also observed in patients included in the BELIEF trial [28], in which longitudinal monitoring of *EGFR* mutations in plasma samples was correlated with clinical responses. However, none of these studies analysed the predictive value of ctDNA clearance after treatment in conjunction with pre-treatment ctDNA levels. In this way, we believe that future studies—whether with sequential TKIs, with TKIs plus chemotherapy or with antiangiogenics—should include quantification of MAF at diagnosis and during patient follow-up for optimal use of the available treatment options. In other words, adding a 3-month determination could inform decisions about whether switching treatment or introducing another drug is warranted [29], thus enabling reliable selection of patients for sequential TKI treatment.

Finally, detection of the T790M resistance mutation is an important factor for guiding clinical decisions on disease progression. The resistance mutation was only

detected in one of the diagnostic samples versus 52.3% of samples taken at progression. Other groups have also reported a T790M detection rate at progression of 50% [30]. As expected, the percentage of T790M detection in our study increased over time, with 3.2% of samples being positive at 3 months and 14.8% of samples being positive at 6 months. Patients with T790M mutation detected in plasma in the 6-month evaluation sample had a high risk of disease progression and death. Serial T790M determination throughout treatment can detect greater incidence of resistance mutations and could justify variation in the different series [31]. Moreover, if it is decided to initiate treatment with first- or second-generation TKIs, early detection of the resistance mutation is possible, allowing a subsequent sequential treatment with osimertinib.

Among those patients with T790M mutation detected in the sample at progression, a significant association with smoking habits (64.3% were never-smokers) and metastatic sites at diagnosis (57.1% had bone metastases, and 26.5% had liver metastases) was observed. However, because there is no doubt that upfront osimertinib ensures prolonged PFS, better predictors for the appearance of the T790M mutation are needed to ensure an optimal selection of patients for the sequential approach.

5. Conclusions

We present thorough and longitudinal analyses of *EGFR* mutation levels in liquid biopsies from patients with *EGFR*-positive NSCLC treated with a TKI. Our results support the usefulness of ctDNA to identify patients with *EGFR*-positive NSCLC at low risk of disease progression and death who could benefit from sequential TKI treatment.

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Author contributions

M.P. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. M.P., A.R., F.F. and V.C. contributed to concept, design and project administration. M.P. and A.R. contributed to funding acquisition. All authors contributed to acquisition, analysis or interpretation of data. M.P., A.R., R.S.-B. and M.A. contributed to investigation and methodology. A.R. and R.S.-B. contributed to statistical analysis. M.P., A.R. and R.S.-B. contributed to original draft, writing review and editing. M.P., A.R., F.F., V.C. and A.R. contributed to critical revision of the manuscript for important intellectual content. M.P. and A.R. contributed to supervision, validation and visualisation.

Conflict of interest statement

M.P. reports personal fees from Roche, BMS, MSD, Pfizer, Lilly, Novartis and Takeda grants and personal fees from AstraZeneca and Boehringer during the conduct of the study. V.C. reports personal fees from Roche, BMS, MSD, Pfizer, Lilly, AstraZeneca, Boehringer, Novartis and Takeda, during the conduct of the study. A.S.-H. reports personal fees and non-financial support from Roche, AstraZeneca and Boeringher, outside the submitted work. M.d.J.C. reports personal fees from Roche, Pierre Fabre and MSD and other from AstraZeneca, Boehringer Ingelheim, AstraZeneca and Lilly, outside the submitted work. M.D. reports personal fees from AstraZeneca, BMS, Boehringer Ingelheim, MSD, Pfizer and Roche, outside the submitted work. J.B.-B. reports grants and personal fees from Roche-Genentech, Pfizer and Pierre Fabre and personal fees from MSD, BMS, AstraZeneca and Novartis. A.P. reports personal fees and non-financial support from AstraZeneca, Hoffmann-La Roche, BMS, MSD, Novartis and EUSA Pharma, outside the submitted work. C.B. reports personal fees from Novartis and Roche, outside the submitted work. E.J.-L. reports personal fees from Amgen, AstraZeneca and BMS, outside the submitted work. A.R. reports personal fees from Boehringer and Takeda. The rest of the authors have declared no conflict of interest.

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Appendix A. Supplementary data

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