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From the beginning to resistance: Study of plasma monitoring and resistance mechanisms in a cohort of patients treated with osimertinib for advanced T790M-positive NSCLC

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ABSTRACT

Introduction: Analysis of circulating tumor DNA (ctDNA) for the identification of T790M mutation in advanced *EGFR*-mutated NSCLC patients can replace tissue re-biopsy for resistance characterization and, being non-invasive, may be applied for disease monitoring. We analysed ctDNA during osimertinib treatment to correlate mutational levels with clinical outcome and to predict pattern of resistance.

Materials and Methods: Forty patients with advanced NSCLC receiving osimertinib for T790M + disease after previous EGFR-TKI were enrolled in a pilot study to collect plasma at baseline and every 12 weeks until progression. Molecular analysis of ctDNA was performed by ddPCR and Therascreen®. When feasible at progression, tissue re-biopsy and NGS analysis were performed.

Results: Thirty-eight patients had baseline plasma samples suitable for molecular analysis. Patients with low levels of the *EGFR* activating mutation in ctDNA [< 2200 copies/mL or allele frequency (AF) < 6.1%] showed better progression-free survival (17.8 or 17.8 months vs. 4.3 or 2.7, p = 0.022 or p = 0.018, respectively) and overall survival (23.6 or 23.6 vs. 7.7 or 7.3, p = 0.016 or p = 0.013, respectively) than patients with high levels (≥ 2200 copies/mL or AF ≥ 6.1%). Patients with detectable *EGFR* mutations in plasma (*shedders*) presented worse outcome than negative subjects (*non-shedders*). Low levels of T790M, higher T790M/activating mutation ratio and complete clearance after 2 months were associated with a trend towards better outcome. Tissue re-biopsy at resistance showed 3 patients with *EGFR* C797S, 1 with *MET* amplification, 1 with *MYC* amplification, 1 with *PTEN* loss, 3 with SCLC transformation.

Conclusions: The mutational analysis performed on plasma plays a significant role in prognostic stratification, especially for the *EGFR* activating mutation, since patients with absence or low levels of mutations presented a better outcome to osimertinib. At progression, tissue re-biopsy remains a crucial issue for the identification of resistance mechanisms.

1. Introduction

Activating mutations of the Epidermal Growth Factor Receptor ($EGFR^{MUT}$) gene are found in approximately 10–12% of Caucasian patients affected by non small-cell lung cancer (NSCLC), with higher prevalence in adenocarcinoma histology, never smokers and females [1]. Exon 19 deletions and exon 21 L858R point mutation represent about 90% of these activating alterations [2]. For many years, first- (gefitinib and erlotinib) or second-generation (afatinib) EGFR-tyrosine kinase inhibitors (TKIs) have been considered the standard first-line

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therapy for patients with advanced *EGFR*-mutated NSCLC, based on the excellent outcome in comparison with standard chemotherapy [1]. Nevertheless, resistance inevitably develops and, in approximately 50% of patients, it is due to the appearance of the secondary mutation T790M in exon 20 [3].

Osimertinib is a third-generation EGFR-TKI specifically designed to target T790M-positive *EGFR* in patients who have developed resistance to earlier generation drugs [4]. Osimertinib significantly improves clinical outcome in these patients with respect to chemotherapy [5], but, again, resistance may develop and includes additional *EGFR* mutations (mainly C797S), *KRAS*, *PIK3CA* and *BRAF* V600E mutations, *MET* and *HER-2* amplifications, oncogenic fusions in *FGFR3*, *RET* and *NTRK* and small-cell transformation [6–8].

Conventionally, a tissue biopsy should be performed to evaluate acquired resistance mechanisms [1]; however, re-biopsy has several limitations, considering that is an invasive procedure, not always feasible and accepted by patients, and that it does not account for the heterogeneity in resistance mechanism expressed at different tumour sites in the same patient. The use of circulating cell-free DNA (cfDNA) extracted from plasma to detect genetic alterations, also known as liquid biopsy, represents a non-invasive option and may be used to detect mechanisms of resistance to EGFR-TKIs avoiding tissue re-biopsy. Moreover, molecular analysis of plasma collected during the course of the disease allows *on-treatment* monitoring of disease response.

To date, if a liquid biopsy is performed to detect the T790M, there is no way to demonstrate a sure predictive role about osimertinib treatment outcome. Preliminary results published by our group showed that the allele frequency (AF) of $EGFR^{MUT}$ and T790M/$EGFR^{MUT}$ ratio are potential markers of outcome in patients treated with osimertinib [9]; in particular, patients who achieved a clinical benefit presented a lower $EGFR^{MUT}$ AF and a higher T790M/$EGFR^{MUT}$ ratio at baseline compared to non-responders. Similarly, a significant correlation has been demonstrated on baseline plasma samples (*shedders* vs. *non-shedders*) and outcome measures of patients treated with osimertinib [10].

Moreover, it is still uncertain if the levels of $EGFR^{MUT}$ in circulating tumor DNA (ctDNA) at osimertinib baseline and during treatment could predict the pattern of resistance. Oxnard et al. observed that patients who lost T790M had only a slightly lower relative T790M AF before osimertinib than patients who maintained T790M; moreover, the evaluation of $EGFR^{MUT}$ levels demonstrated a larger decrease for patients who maintained T790M vs. T790M loss [11].

Therefore, the present study analysed the cfDNA of advanced NSCLC patients treated with osimertinib to confirm the correlation of plasma levels of $EGFR^{MUT}$ and T790M with clinical outcome, and to predict the pattern of resistance at tumor progression.

2. Materials and methods

2.1. Treatment and tissue/plasma sampling

ASTRIS (NCT02474355) trial was an open-label, multinational, multicenter, real-world treatment study of osimertinib for patients with advanced *EGFR* T790M-positive NSCLC progressed to prior first-/second-generation EGFR-TKI. Patients were considered eligible if T790M was detectable in either plasma or tissue biopsies. Patients enrolled in the ASTRIS trial at the University Hospital of Parma were also asked to participate in a pilot study of ctDNA monitoring before and during treatment with osimertinib. Plasma samples were collected before the beginning of treatment (baseline sample) and every 12 weeks, at the same time of radiological assessment planned as per ASTRIS protocol, until disease progression. If technically feasible and clinically indicated, a tissue re-biopsy after progression to osimertinib was proposed to patients to study possible resistance mechanisms and correlate them with mutations detected in cfDNA. The pilot study of plasma monitoring obtained the ethical approval by local Ethics Committee (the DiNAmic study, EudraCT number: 2018-004309-25) and all patients signed specific Informed Consent Form before any procedure.

2.2. Analysis of EGFR mutational status

Baseline $EGFR^{MUT}$ in tissue were assessed as part of diagnostic procedure by validated methods including Sequenom (Diatech Pharmacogenetics®, Jesi, Italy) or Therascreen EGFR RGQ real-time PCR assay (Qiagen®, Valencia, CA).

The analysis of $EGFR^{MUT}$, T790M and C797S was performed on cfDNA obtained from plasma samples. Six ml of blood were collected in EDTA and centrifuged twice for 10 min at 2000*g* within one hour after blood drawing; plasma samples were stored at −80 °C until analysis. cfDNA was extracted using the QIAmp Circulating nucleic acid kit (Qiagen®, Valencia, CA) from 1 to 3 ml of plasma and the DNA was finally eluted in 50 µl of buffer. The analysis of EGFR activating and resistance mutations on cfDNA was performed by a QX100 digital droplet PCR (ddPCR) platform, using the ddPCR Mutation Assays (BioRad®, Hercules, CA) as previously described [12]. Analyses of $EGFR^{MUT}$ and T790M at baseline and at first evaluation were also performed with Therascreen EGFR RGQ PCR kit (Qiagen®, Valencia, CA), a real time PCR technique, as a comparative analysis.

All the procedures for the molecular analysis have been performed following the specific manufacturer's instructions.

2.3. Next-generation sequencing analysis

Next-Generation Sequencing (NGS) of biopsies at clinical resistance was performed using different NGS assays available as per clinical practice: 3 patients were studied using the TruSight Tumor 26 genes (Illumina®, San Diego, CA), 3 with the TruSight Tumor 170 genes (Illumina®, San Diego, CA) and 2 with the Oncomine Focus Assay (Life Technologies®, Carlsbad, CA). The 3 patients with SCLC transformation, observed with routine histological examinations, were not sequenced with NGS (see supplemental data).

2.4. FISH analysis

FISH (Fluorescent In Situ Hybridization) assay was performed on histological or cytological samples at osimertinib progression (see supplemental data). *MET*, *HER-2*, *EGFR* and *FGFR1* amplifications were evaluated as potential mechanism of acquired resistance. Samples were classified as FISH positive following specific guidelines [13–16].

2.5. Statistical analysis

Fisher exact test, Mann-Whitney test and Chi-square test were used to correlate plasmatic mutations levels and tumoral response. Patients with detectable $EGFR^{MUT}$ in plasma with or without T790M mutation were defined *shedders* while *non-shedders* were subjects who tested negative.

Progression-free survival (PFS) was defined as the duration between osimertinib initiation and progression of disease or death for any cause, whichever occurred first. Similarly, overall survival (OS) was calculated from the date of starting osimertinib and death for any cause or last follow-up (censored patient). The Kaplan-Meier method was employed to estimate survival outcomes (PFS, OS) and curves were compared by using log-rank test. Also, ROC (Receiver Operating Characteristic) curves were created for PFS in order to identify a cut-off value with the best predictive performance for progression. In case of ROC cut-off with high deviance from the calculated median value, we considered this last for statistical purpose. When calculating median values or ROC cut-offs for quantitative analyses performed with ddPCR, patients with no detectable mutations were excluded.

Tumor response was evaluated according RECIST criteria version 1.1. Cut-off for clinical data collection was 15th July 2018. Statistical analysis was done with SPSS v25 (IBM Corporation, NY, USA).

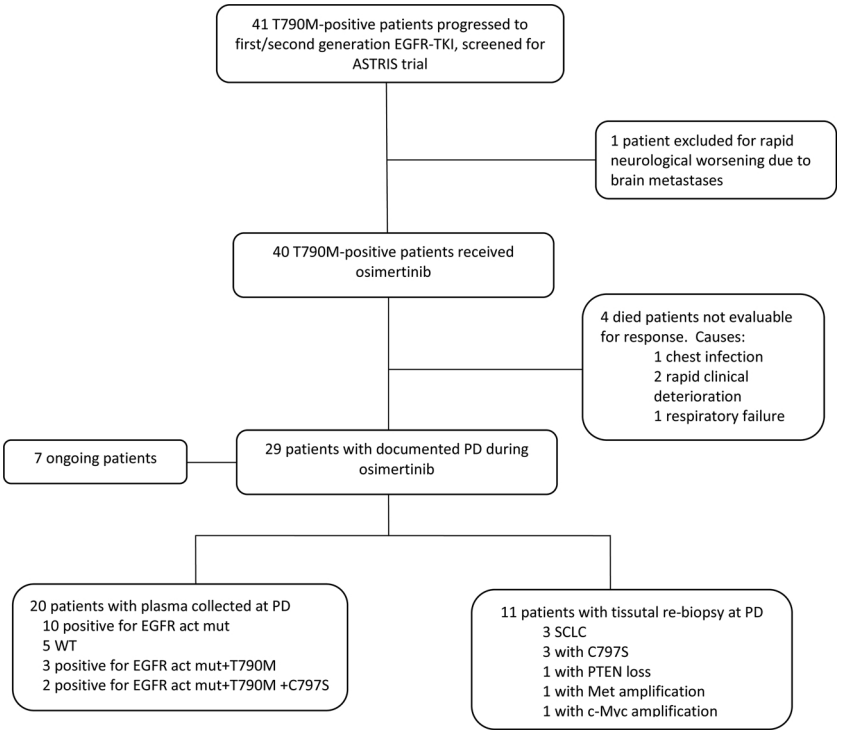

Fig. 1. Flow-chart of first-line EGFR-TKI resistant patients screened for ASTRIS trial and included in the pilot study of plasma monitoring during treatment with osimertinib.
Abbreviations: TKI, tyrosine kinase inhibitor; PD, progressive disease; SCLC, small-cell lung cancer; act, mutactivating mutation; WT, wild-type.

3. Results

3.1. Patient's characteristics

From October 2015 to December 2016, 41 patients were screened to be enrolled in ASTRIS clinical trial at our institution. Only one patient was excluded, due to rapid neurological deterioration (Fig. 1). Characteristics of enrolled patients are summarized in Table 1. Common activating alterations were largely represented; in one patient both activating (ex19del) and T790M were found at baseline.

During first-line treatment, overall response rate (ORR) and PFS resulted 90% and 12 months, respectively. After progression, 77% of patients received osimertinib, whilst 23% of patients was treated with chemotherapy and started osimertinib after new documented progression. However, as per protocol, before starting osimertinib, T790M mutation was confirmed in all patients: in 24 of them (60%) by means of liquid biopsy and in the remaining 16 (40%) with tissue re-biopsy.

The first patient started osimertinib on 14th October 2015 and the last one assumed the first dose on 12th December 2016. Median follow-up was 26.3 months (95% Confidence Interval, CI: 23.1–29.5 months). The data cut-off for this analysis was 15th July 2018; at that time, 7 patients were still on treatment and 29 had documented progression of disease (PD) during osimertinib (Fig. 1). Four patients were not evaluable for response (Fig. 1). Only 1 patient discontinued permanently osimertinib due to pulmonary toxicity; at the time of interruption the disease was measured as stable (SD) and that was considered the best response. Notably, 2 additional patients had a temporary treatment interruption due to lung toxicity; however, after resolution, treatment was restarted with dose reduction.

In the overall population, median PFS resulted 10.8 months (95%CI 3.1–18.5 months) and median OS reached 15.3 months (95%CI 13–17.6 months). Tumor responses were classified as follow: 6 patients (17%) and 13 patients (36%) had complete (CR) and partial responses (PR), respectively; 6 patients (17%) presented SD and 11 patients (30%) had PD. Disease control rate (DCR, including CR, PR, and SD) resulted 70%.

Table 1
Patients' baseline clinical characteristics.

Patients' characteristics		No. (%)
Age, median (range)		65 (36 – 87)
Gender	male	11 (27%)
	female	29 (73%)
ECOG PS	0-1	33 (83%)
	2	7 (17%)
Smoking history	Never	25 (63%)
	Former smokers/ smokers	15 (37%)
Stage at diagnosis	IV/relapsed	38 (96%)
	IIIB	2 (4%)
Histology at diagnosis	adenocarcinoma	40 (100%)
EGFR activating mutation	ex19del	30 (75%)*
	L858R	9 (23%)
	S768I	1 (2%)
First line EGFR-TKI	gefitinib	23 (58 %)
	erlotinib	10 (25%)
	afatinib	7 (17%)
First line EGFR-TKI outcome	ORR	35/39 (90%)**
	PFS (months)	12,0
Post-TKI treatment	osimertinib	31 (77%)
	chemotherapy	9 (23%)***
Re-biopsy at resistance to first-line TKI for T790 M confirmation	Tissutal (including citology)	16 (40%)
	Liquid (plasma)	24 (60%)

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group Performance Status; TKI, Tyrosine Kinase Inhibitor; ORR, Overall Response Rate; PFS, Progression Free Survival.

* one patient diagnosed with EGFR ex19del and T790M point mutation ex novo.

** only 39/40 patients evaluable for response (1 patient discontinued TKI due to pulmonary toxicity).

*** all patients received osimertinib after progression to chemotherapy.

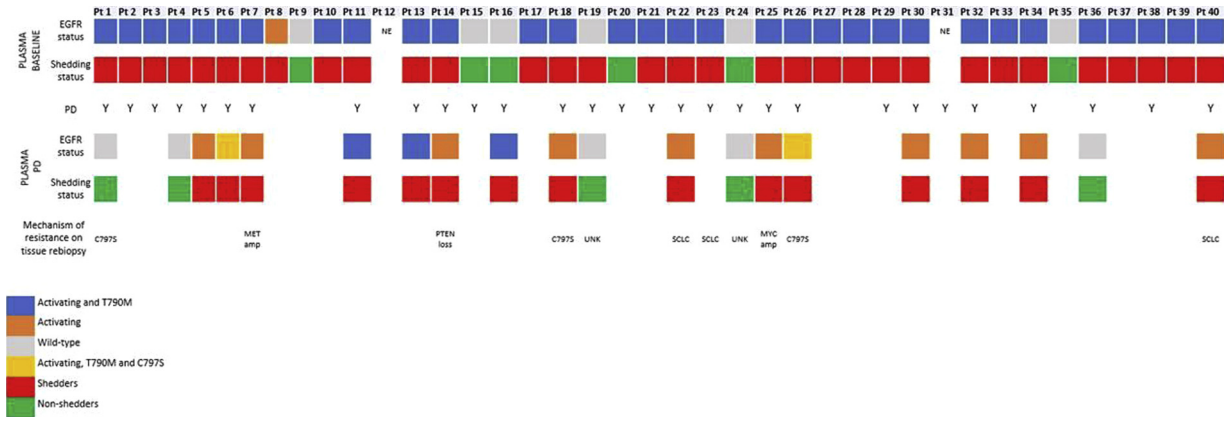

Fig. 2. Plasmatic molecular pattern at baseline and progression and results of tissue re-biopsy after progression to osimertinib.
Abbreviations: NE, not evaluable; PD, progressive disease; Y, yes; UNK, unknown; SCLC, small-cell lung cancer.

In supplemental data, the results of prognostic factors were reported.

3.2. cfDNA analysis

3.2.1. cfDNA at baseline

At baseline, plasma for cfDNA analysis by ddPCR and Therascreen was available from 38 out of 40 patients; 32 patients had the $EGFR^{MUT}$ (ex19del, n = 24 and L858R, n = 8; Fig. 2). Six patients were *EGFR* wild-type, including the patient with S768I, which was not analysed by ddPCR. Overall, baseline median value of $EGFR^{MUT}$ in the group of 32 patients was 4910 copies/mL and the corresponding median AF was 9.8%. Similarly, when analysed for T790M mutation, 31 patients resulted positive and 7 were negative in baseline samples. Median levels for baseline T790M mutation were 590 copies/mL, corresponding to 1.4% of median AF. Of note, the patient diagnosed with baseline T790M mutation in tissue, resulted wild-type for both activating and resistance mutations examined by ddPCR. In our cohort, the median baseline ratio between allele frequencies of T790M mutation/activating mutation resulted 0.25.

Regarding the analyses by Therascreen, only 24 patients were positive for the $EGFR^{MUT}$ (16 with ex19del and 8 with L858R) and the majority (n = 25) were negative for T790M mutation. In particular, the group of patients positive for $EGFR^{MUT}$ at Therascreen analysis showed higher levels (median AF, 20.44%) than wild-type one (median AF of 3.54%; p = 0.001) (Table S1).

No correlation emerged between levels of ctDNA mutations and clinical parameters including age, gender, number or type of site of metastases (data not shown). Several correlations were found between levels of mutations and outcome measures. In particular, baseline plasmatic levels of $EGFR^{MUT}$ demonstrated correlations with DCR, PFS and OS.

Patients with lower amount of mutations (< 2200 copies/mL, cut-off generated by means of ROC analysis) showed better PFS (17.8 vs. 4.3 months, p = 0.022, Fig. 3a) and OS (23.6 vs. 7.7 months, p = 0.016, Fig. 3b). However, no differences emerged in DCR according to *EGFR* baseline copies. These findings were confirmed also considering baseline AF of $EGFR^{MUT}$; in fact, median PFS was 17.8 vs. 2.7 months (p = 0.018, Fig. 3c) and median OS 23.6 vs. 7.3 months (p = 0.013, Fig. 3d) if AF < vs. ≥ 6.1%, respectively (cut-off determined with ROC analysis). Patients with AF < 6.1% presented higher probability of DCR than patients with AF ≥ 6.1% (83% vs. 55%, p = 0.235). Based on detection of $EGFR^{MUT}$, *non-shedders* had higher DCR (80% vs. 67%), longer median PFS (19.5 vs. 3.7 months) and longer OS (median Not Reached vs. 15.2 months) than *shedders*, even if differences were not statistically significant (Fig. 3e, 3f). Similar analysis was conducted considering Therascreen data; differences favoured patients with negative plasma and were statistically significant (supplemental data and Fig. S1).

Then, we analysed outcome differences considering baseline levels of T790M. Patients with T790M AF < 1.4% or with T790M copies/mL lower than median value presented better DCR, PFS and OS compared to patients with higher levels of T790M AF or copies/mL, even if differences were not statistically significant (supplemental data and Table S2).

The ratio between median AF T790M/$EGFR^{MUT}$ ≥ 0.25 (ROC cut-off) discriminated patients with better PFS, OS and better DCR, but all these differences were not significant (supplemental data and Table S2). However, we observed that the ratio was significantly higher in patients with disease control than patients with PD as best response (p = 0.02, Fig. 4).

3.2.2. cfDNA during osimertinib treatment and at progression

Using ddPCR, we evaluated the plasmatic mutation levels after 12 weeks of osimertinib. Twenty-three plasma samples were analysed: 8 patients negative at baseline were excluded *a priori* together with 9 patients unavailable for further plasma sampling, mainly due to clinical deterioration and death. Ten patients had plasma clearance of mutations, 11 patients had persistence of $EGFR^{MUT}$ and 2 patients had both $EGFR^{MUT}$ and resistance mutation detectable in plasma. Patients with complete clearance presented higher DCR (80% vs. 61.5%, p = 0.41), better PFS (17.9 vs. 11 months, p = 0.13) and OS (18.5 vs. 16.2 months, p = 0.77) than patients with at least one plasmatic mutation.

Twenty-nine patients experienced progression of disease to osimertinib. *EGFR* status was analysed at PD in 20 out of 29 patients with available plasma sample (Fig. 1 and 2). Regarding the shedding status, 5 were non-shedders while 15 were shedders (5 with both $EGFR^{MUT}$ and T790M and 10 with $EGFR^{MUT}$ only). The analysis of *EGFR* C797S revealed the presence of the mutation in 2 patients only, which were also positive for $EGFR^{MUT}$ and T790M mutations: in one case, C797S was detectable 2 months before progression (Fig. S2 panel A, patient #26) whilst in the other case the tertiary mutation was detected only at the timing of the PD. Other results about the shedding status were reported in supplemental data.

3.3. Resistance mechanisms to osimertinib treatment

Post-progression tissue biopsy was collected in 11 of 29 patients. The analysis of putative mechanism of resistance revealed that 3 patients presented *EGFR* C797S tertiary mutation (2 patients with c.2389A > T and 1 with both c.2389A > T and c.2390 G > C), while the activation of by-pass signalling pathways was observed in 3 patients: 1 with *MET* amplification, 1 with *MYC* amplification and 1 with *PTEN* loss. SCLC transformation was observed in 3 patients (as described previously [8]), while in 2 patients no mechanisms of resistance

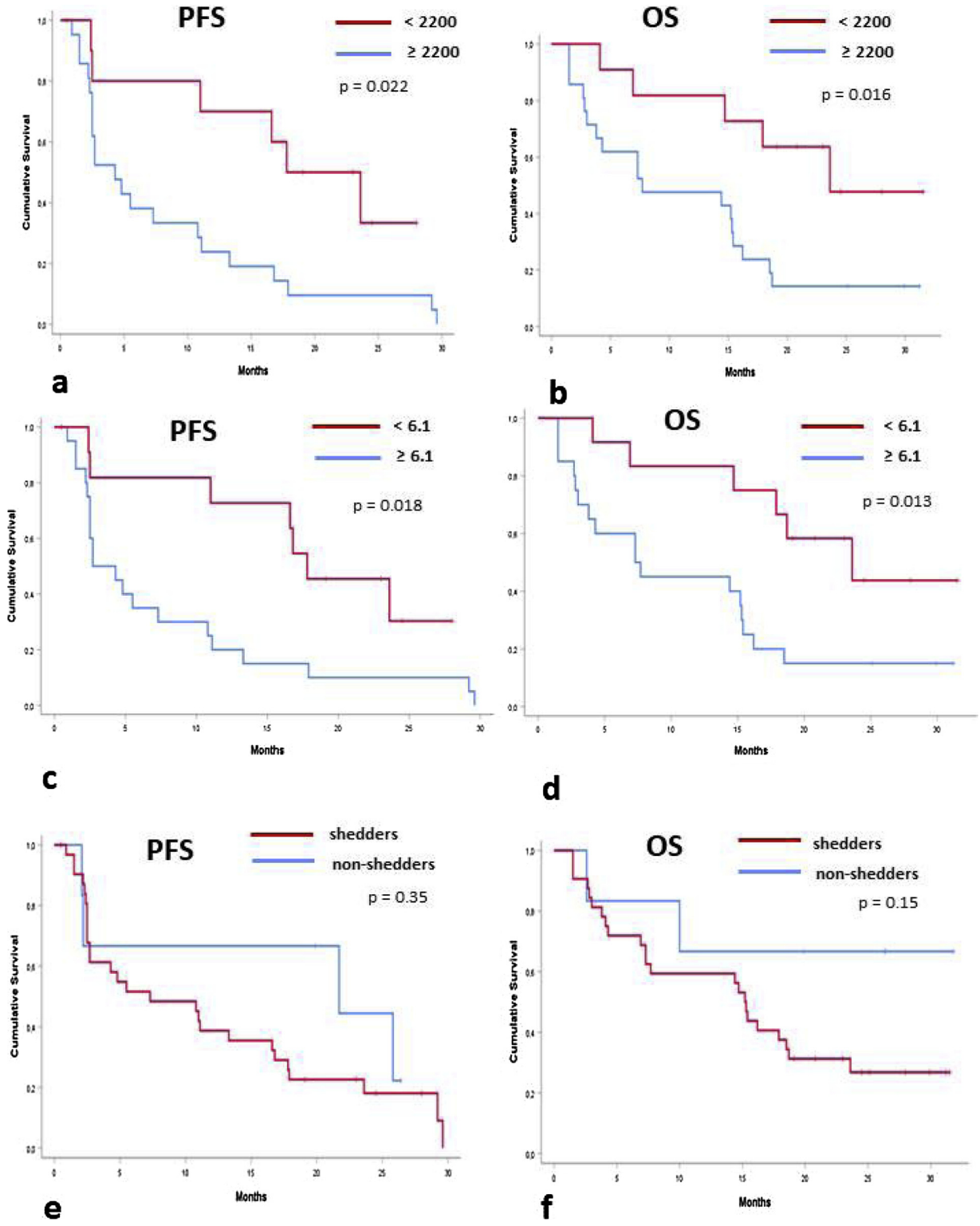

Fig. 3. Survivals according to baseline plasmatic molecular status by ddPCR. (A) PFS according to *EGFR*^{MUT} levels, copies/mL; (B) OS according to *EGFR*^{MUT} levels, copies/mL; (C) PFS according to *EGFR*^{MUT} levels, median allele frequency; (D) OS according to *EGFR*^{MUT} levels, median allele frequency; (E) PFS according to shedding status; (F) OS according to shedding status. *Shedders,* patients with detectable *EGFR*^{MUT} in plasma with or without T790M mutation; *non-shedders,* patients with plasma sample negative for any EGFR mutation.

to osimertinib were detected (Table 2). Patients with SCLC transformation and the patient with *MYC* amplification presented a primary resistance to osimertinib (Table 2). T790M mutation was confirmed in tissue at resistance only in C797S positive patients while other subjects displayed a T790M loss. The difference, in terms of PFS, between two subgroups lost vs. maintained T790M was not statistically significant (median 10.3 vs. 11.4 months).

These re-biopsied patients, at baseline, presented detectable *EGFR*^{MUT} and T790M mutations in cfDNA, except two, who resulted wild-type at all time points evaluated. At first plasma monitoring, all

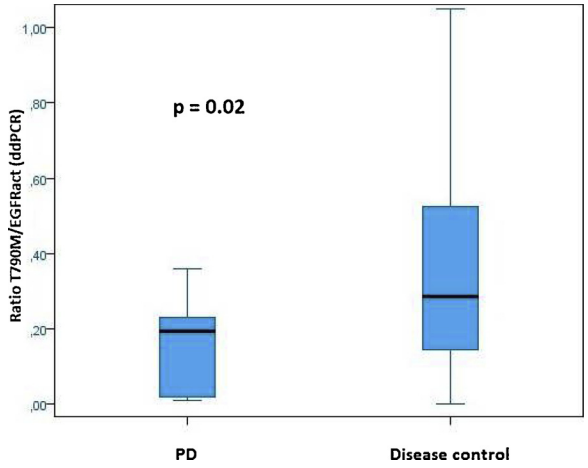

Fig. 4. Distribution of ratio (T790M/*EGFR*^{MUT}) values in baseline plasma samples according to osimertinib response.
Abbreviations: PD, progressive disease; ddPCR, digital droplet PCR.

Table 2
Results of performed tissutal re-biopsies after progression to osimertinib. Mechanisms of resistance are divided according to persistence of T790M positivity or not and corresponding outcome to osimertinib is reported.

Resistance to osimertinib							
T790M +				T790M-			
Pt	Mechanism	PFS	Best Response	Pt	Mechanism	PFS	Best Response
#1	EFGR C797S, c.2389 T > A	16.6	PR	#7	MET amp	29.2	CR
#18	EFGR C797S, c.2389 T > A	11.4	PR	#14	PTEN loss	17.9	PR
#26	EFGR C797S, c.2389 T > A c.2390 G > C	11.1	SD	#19	n.d.	21.7	PR
				#22	SCLC	2.7	PD
				#23	SCLC	0.9	PD
				#24	n.d.	25.8	PR
				#25	MYC amp	2.7	PD
				#40	SCLC	2.3	PD

Abbreviations: PFS, progression free survival (months); PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; n.d., not detectable.

patients became negative for T790 M mutation and, regarding *EGFR*^{MUT}, it was strongly reduced or disappeared with the exception of SCLC transformation, who showed a strong increase in *EGFR*^{MUT} (Fig. S2 panel B, patients #22 and #40). At progression, C797S was detected in 1 out of 3 patients with tissue biopsy (Fig. S2 panel A, patient #26), as described above, whilst the other two resulted wild-type (Fig. S2 panel A, patient #1) and positive only for *EGFR*^{MUT} (Fig. S2 panel A, patient #18). Patients with *MET* amplifications and *PTEN* loss were negative for T790M in plasma at progression; however, they presented increasing levels of *EGFR*^{MUT} (Fig. S2 panel C, patients #7 and #14). Interestingly, the 2 patients with unknown putative mechanism of resistance were wild-type in all collected samples.

4. Discussion

Our study aimed to investigate the role of plasma monitoring during osimertinib therapy in patients with advanced T790M-positive NSCLC resistant to prior EGFR-TKI. Forty patients were enrolled and treatment outcomes were prospectively recorded whilst plasma was collected before and during treatment until progression of disease. Overall, outcomes of our study population reflect data reported in literature in this setting [5]; response rate was slightly inferior to what expected due to the heterogeneity of resistance mechanisms (i.e. SCLC transformation) likely present together with T790M mutation, as previously reported [8].

Plasma samples were analysed using both ddPCR and Therascreen, allowing us to obtain several parameters with quantitative or qualitative significance to describe *EGFR* mutational status. The most reliable predictive factor was *EGFR*^{MUT} expressed at baseline. In particular, the quantification of the amount of activating mutations in copies/mL, performed with ddPCR, confirmed that higher levels are associated with poor outcome. This observation was strengthened when analyses were conducted considering median AF. Other authors described inverse relationship between activating mutations levels and outcome [17]. Recently, our group published results from a cohort of patients treated with osimertinib with *EGFR*^{MUT} and resistance mutations detectable in plasma and documented a significant improvement in clinical outcome in patients with lower levels of *EGFR*^{MUT} AF [9]. The present new analysis included all patients enrolled in the ASTRIS trial at the University Hospital of Parma, and allowed also a comparison between shedders vs. non-shedders, highlighting a trend favouring patients with undetectable mutations in plasma. The real-time PCR approach does not provide quantitative information about ctDNA and its sensitivity is inferior to ddPCR: in our study population, the median AF of activating mutations determined by ddPCR was 3.5% in patients resulted negative by real-time PCR vs. median AF 20.4% in patients positive by real-time PCR ($p = 0.001$). Therascreen allowed a selection of patients with highly-positive plasma samples with a statistically significant poorer disease control and survival, with respect to negative patients. Our findings are confirmed in other reports evaluating the prognostic impact of cfDNA plasma positivity [10,18]. A possible explanation could be found in the correlation between ctDNA quantification and tumor burden, historically considered a clinical feature of poor prognosis [19]. Taken together, the previous and present studies reinforce the prognostic significance of plasmatic mutational analysis in patients candidate to osimertinib, deserving further in depth analysis especially in first-line setting perspective.

Our study underlines the poor reliability of T790M quantification by ddPCR for prognostic purpose and similar results have been reported by other authors [9,11,17]. A possible explanation can be argued considering the heterogeneous context determined by different resistant clones selected during first-line EGFR-TKI, that share the *EGFR*^{MUT} as molecular driver. In fact, we previously documented that the amount of T790M mutation is notably inferior than *EGFR*^{MUT}, suggesting that T790M determination can not reflect the molecular complexity of different sub-clones [20]. Moreover, in this study we observed that patients with SCLC transformation had a disappearance of T790M in plasma despite disease progression, again confirming that T790M level cannot represent a reliable marker for response. Our results are confirmed by current literature, which underscores the weakness of prognostic role of T790M alone [9,21,22].

The prognostic role of the ratio between median AF T790M to median AF of *EGFR*^{MUT} was also investigated, confirming the results obtained by other authors, that a higher ratio is associated with deeper tumor shrinkage and better survival [21,23,24]. In fact, the ratio of T790M-to-*EGFR*^{MUT} could serve as quantification of the T790M-positive clones in relation to other resistant clones still carrying the *EGFR*^{MUT} but no T790M, providing a more accurate stratification with respect to absolute level of T790M.

Moreover, we observed a trend towards better outcome in patients with clearance of plasmatic mutations during osimertinib treatment, as recently confirmed in literature [25,26]. Only two of the evaluable patients presented both *EGFR*^{MUT} and T790M mutation at the first plasma sample collected during osimertinib. Therefore, the trend observed in our population could have been mainly driven by differences between patients with total clearance and patients with persistence of

EGFR^{MUT}. Similarly to us, Oxnard et al observed that the pattern of tissue resistance could not be predicted by T790M plasmatic behaviour neither at baseline nor during therapy. On the contrary, they showed a different plasmatic behaviour of *EGFR*^{MUT} in the two groups of patients with T790M-loss or persistence at resistance, observing that patients with T790M-loss are more likely to present lower decrease of the *EGFR*^{MUT}, representing sub-clones less sensitive to osimertinib and candidate to drug resistance. Thus, our observation of better outcome in patients with complete clearance finds support in emerging literature even if, due to the small number of patients, we were not able to confirm the predictive role for the tissue resistance mechanisms.

To study mechanisms of resistance, tissue re-biopsy at progression to osimertinib was performed in 11 patients. Even though the small number of patients analysed and the limitations due to different platforms used for NGS analyses, qualitative considerations could be taken. Recently, Papadimitrakopoulou et al presented data about resistance mechanisms in patients enrolled in the AURA3 trial well summarizing the complex scenario [7]. In our cohort, 8 out of 11 patients (72%) lost T790M and this observation slightly higher than the percentage described in literature (34–68%) in the most recent cohorts analysed [10,27–31]. Loss of T790 M at progression was associated with worse outcome [27]. In our cohort the difference, in terms of PFS, between two subgroups lost vs. maintained T790M was not statistically significant. Resistance to osimertinib by reactivation of EGFR pathway through tertiary mutations was observed in 3 patients that showed the emergence of *EGFR* C797S (27%). Activation of known by-pass signalling pathways as mechanism of resistance was represented in our cohort by MET (1 patient, 9%) and MYC amplification (1 patient, 9%), and PTEN loss (1 patient, 9%). MET amplification, observed in our study in only one case with loss of T790M, has been described in the literature at frequencies ranging 5–50% [10,27–31]. PTEN loss was already mentioned as a possible mechanism of resistance to osimertinib [31], while MYC amplification is described here, for the first time to best of our knowledge, as potential mechanism of intrinsic resistance. In our cohort, a higher percentage of SCLC was observed (3 patients, 27%) than previously reported in other studies on the same topic (4–15%) [10,27–31]. All of SCLC transformed patients were enrolled in the ASTRIS trial only based on liquid biopsy and developed a primary resistance to osimertinib. The plasmatic information of T790M may underestimate the coexistence of different mechanisms of resistance at the moment of progression of disease to first/second-generation EGFR-TKI [8].

After publication of results from the FLAURA trial [32] and considering the high probability for osimertinib to move into front-line setting, a huge effort is underway to identify possible predictors of treatment efficacy and to stratify patients beyond *EGFR*^{MUT} positivity. Our study, with a limitation due to the small sample size and of some data already published [8,9], confirms the importance of testing plasma at baseline for *EGFR*^{MUT}, as patients with absence or low levels of *EGFR*^{MUT} are significantly associated with better outcome. Moreover, we confirm the *EGFR*^{MUT} tested in plasma as a surrogate biomarker for prognosis and response prediction. Given the heterogeneity of resistance mechanisms to third-generation TKI, tumor re-biopsy after progression on osimertinib remains a crucial issue for understanding tumor biology and to offer patients the best therapeutic options.

Conflict of interest statement

No potential conflicts of interest to disclose.
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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.03.017.

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