



Liquid biopsies: envisioning a future when tissue is avoidable in lung cancer treatment decision-making

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During the last years we have witnessed a revolution in the genomic profiling and molecular characterization of lung cancer. Epidermal growth factor receptor (*EGFR*) is, among other signaling pathways such as *ALK*, *BRAF*, *MET* or *ROS*, one of the key oncogenes with potential for targeted-inhibition. Therefore, a complete basal genomic profiling is now recommended in the clinical diagnostic workup to select the optimal personalized therapy for each lung cancer patient (1).

Treatment with first- and second-generation EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib, gefitinib and afatinib, are currently considered the standard of care at first-line in patients with sensitive mutations (Exon 19 Deletions, *L858R*) at the kinase domain of the *EGFR* gene (1). Despite the initial outstanding responses, patients ultimately progress after a median of 9–12 months. One of the most common mechanism of resistance described is the threonine-to-methionine substitution at amino acid position 790 (*T790M*) in exon 20 of the *EGFR* gene that occurs in approximately 50–60% of patients. Other less frequent bypass track mechanisms have also been reported such as the activation of *MET*, *HER2*, *AXL*, *IGF1R*, or *FGFR1* (2).

Osimertinib (AZD9291) is an oral, selective third-generation, irreversible EGFR TKI with activity against both EGFR-TKIs sensitizing and *T790M* resistance mutations (3). In November 2015 and April 2016, the US Food and Drug Administration (FDA) (4) and the European Medicines Agency (EMA) (5) respectively, approved the use of osimertinib at the recommended 80 mg daily dose, in advanced non-small cell lung cancer (NSCLC) patients harboring a *T790M* mutation whose disease progressed on

EGFR-TKI therapy, based on the data of the expansion cohort of the phase I study (namely AURA) with osimertinib and two phase II studies (AURA extension and AURA 2). The results reinforced the efficacy of osimertinib, in *T790M*-mediated resistant patients, with remarkable overall response rate (ORR) (66–71%), encouraging progression-free survival (PFS) (9.7–11 months) and a manageable safety profile (6).

The confirmatory trial was a randomized, phase III trial (AURA3) in TKI-resistant, EGFR *T790M*-positive and advanced NSCLC patients. Osimertinib significantly improved PFS (10.1 *vs.* 4.4 months; hazard ratio 0.30; *P*<0.001) and ORR (71% *vs.* 31%; *P*<0.001) with better toxicity profile (adverse events $\geq$ grade 3: 23% *vs.* 47%) and QoL as compared with standard platinum-pemetrexed chemotherapy (7).

However, all this innovation in terms of effective treatments and new molecular knowledge in the field of EGFR NSCLC represents a clinical challenge because at disease progression, a new tumor re-biopsy is endorsed in order to identify the underlying mechanism of resistance (1). In turn, this has become a major matter of concern as small biopsies or cytological specimens are the only source of material in almost 80% of NSCLC patients (8) and tumor sampling often requires invasive procedures that might be associated with a higher risk of complications and an increase in the average cost per patient (9). Moreover, genetic results from a single tumor-biopsy do not mirror tumor heterogeneity or allow for monitoring molecular resistance changes along time (10).

In the last years circulating-free tumor DNA (ctDNA),

also known as “liquid biopsy”, has emerged as a sensitive and feasible, non-invasive and cost-effective alternative to tissue biopsies to screening for genetic drivers in advanced NSCLC patients. New platforms using blood-based differ in terms of sensitivity, specificity, turnaround time and the rate of mutation detection. Amplification refractory mutation system (ARMS), Cobas and peptide-nucleic acid (PNA) are non-digital polymerase chain reaction (PCR)-based platforms with semi-quantitative ability for mutant ctDNA analysis. Newer platforms, including droplet digital PCR (ddPCR) and BEAMING (beads, emulsion, amplification and magnetics) PCR, allow to quantify the amount of mutant ctDNA, whilst next generation sequencing (NGS) allow for a more extensive analysis of a range of genomic alterations (11,12).

Updated ESMO and NCCN guidelines (1,13) have incorporated ctDNA testing in their algorithms as an available and complementary technique for *T790M* mutation detection in EGFR-mutant NSCLC at disease progression. Among the different blood-based diagnostic tests, the only one that has recently granted approval by the FDA to aid clinicians in selecting patients with metastatic NSCLC with specific sensitive (exon 19 and 21) and resistant (*T790M*) EGFR mutations is the Cobas EGFR mutation testing platform v2 (Cobas® EGFR test) (14).

The study published recently by Remon *et al.* in *Annals of Oncology* (15), aimed to prospectively assess the efficacy of osimertinib in a cohort of TKI-resistant EGFR-mutant NSCLC patients when *T790M* status was determined solely and exclusively in ctDNA from blood samples by using an Inivata Invision™ enhanced Tagged Amplicon-Sequencing technology (eTA-m-Seq™) (16).

The study included 48 EGFR-mutant advanced NSCLC patients from a single institution between April 2015 and April 2016. Patients had to have advanced NSCLC, a common sensitizing EGFR-mutation (Del19, L858R), clinical or radiological progression to at least one first- or second-generation EGFR TKI and importantly be ineligible for a new tissue re-biopsy for *T790M* resistance screening. Upon response evaluation criteria in solid tumors (RECIST) progression, those patients *T790M*-positive, by means of ctDNA-blood testing, were treated with osimertinib (80 mg daily).

Primary objective was to determine the ORR with osimertinib by RECIST on the basis of a positive *T790M* mutational status from ctDNA. Secondary endpoints included PFS (by radiological or investigator's criteria) as well as the percentage of *T790M* positive identified in blood ctDNA.

In the evaluated cohort, ctDNA *T790M* mutation was

detected in 50% (24/48) NSCLC patients. For the primary analysis, among assessable patients (n=16), osimertinib gave an ORR of 62.5%. With a median follow-up of 8.5 months, median PFS was not achieved (95% CI: 4–not available) and was 66.7% and 52% at 6- and 12-months respectively.

ctDNA *T790M* testing in NSCLC: clinical validation of the Cobas assay

The aforementioned health agencies approval of the PCR-based Cobas assay for *T790M* plasma genotyping in EGFR-mutant NSCLC was prompted by the results of the retrospective analysis from paired blood and tissue samples collected from patients included in pivotal trials of osimertinib (AURA phase I–II trials).

A first assessment using Cobas and BEAMing platforms was made in a cohort of patients (n=72) included in the AURA phase 1 study of osimertinib. The two platforms demonstrated moderate sensitivity (73–81%) and specificity (67–58%) respectively. The ORR in patients positive for plasma *T790M* was almost identical to that of patients positive for *T790M* mutation in paired-tissue samples (59% *vs.* 61%) (17). In a second analysis of the same phase I AURA trial, both plasma and tissue genotyping for *T790M* mutation by BEAMing were analyzed in 216 patients. Sensitivity and specificity of *T790M*-positive detection by ctDNA was 70% and 69% respectively. Of 58 patients with *T790M*-negative by tumor tissue 31% (n=18) were *T790M*-positive by plasma. Of these 18 discordant cases (considered “false positives”), 14 (78%) were confirmed using an alternative plasma assays suggesting a real *T790M* mutation. Both, ORR (63% and 64%) and median PFS (9.7 and 7.9 months) were almost identical in *T790M*-positive patients detected in plasma and tissue respectively. Interestingly, better outcomes than expected were observed in plasma *T790M*-negative patients with an ORR of 46% and median PFS of 8.2 months. Among plasma *T790M*-negative subgroup those with tumor *T790M*-positive, obtained an ORR of 69% and median PFS of 16.5 months (similar results to overall plasma *T790M*-positive population) (18).

A larger cohort of 511 patients with matched plasma and tissue samples were evaluated in the phase II trials (AURA extension and AURA2). In these studies, Cobas EGFR mutation plasma test v2 genotyping platform was employed and compared with both Cobas tumor tissue genotyping and NGS plasma genotyping platforms. ORR in patients with *T790M*-positive by both Cobas tissue and plasma platform

Table 1 Validation assays for plasma EGFR T790M mutation in lung cancer (positive and negative percent agreement between the plasma and tissue tests for detection)

Author	Trial	Sample (n)	Study design	Platform	Analysis	Sensitivity (%)	Specificity (%)
Thress <i>et al.</i> , 2015 (17)	AURA 1	72	R	Cobas; BEAMing	QL*; QN	73; 81	67; 58
Oxnard <i>et al.</i> , 2016 (18)	AURA 1	216	R	BEAMing	QN	70	69
Karlovich <i>et al.</i> , 2016 (21)	–	153	R	Cobas; BEAMing	QL*; QN	64; 73	98
Li <i>et al.</i> , 2016 (22)	–	16	R	NGS	QL	94	NA
Reckamp <i>et al.</i> , 2016 (23)	–	60	R	NGS	QL	93	94
Jenkins <i>et al.</i> , 2017 (19)	AURA phase II	551	R	Cobas	QL*	61	79
Sacher <i>et al.</i> , 2017 (24)	–	180	P	ddPCR	QN	77	63
Wu <i>et al.</i> , 2017 (20)	AURA 3	399	R	Cobas	QL*	51	77

*, considered qualitative and semi-quantitative. R, retrospectively validated assay; P, prospectively validated assay; ddPCR, droplet digital PCR; BEAMing, beads, emulsion, amplification and magnetics PCR; QN, quantitative; QL, qualitative; NGS, next generation sequencing; NA, not available; EGFR, epidermal growth factor receptor; AURA, trial programme with osimertinib in advanced NSCLC.

was similar (64% and 66% respectively). ORR for patients evaluated by plasma NGS was not reported. Sensitivity and specificity for Cobas plasma compared with tissue genotyping was 61% and 79% and compared with orthogonal NGS plasma genotyping assay 93% and 92% respectively. A total of 27 patients considered *T790M*-positive by plasma were *T790M*-negative by tissue test. These discordant cases were assessed further by plasma NGS and 23 (85%) of them were confirmed as positive too (19).

Outcomes of ctDNA *T790M*-positive patients of pivotal AURA 3 cohort have been recently reported. Cobas plasma *T790M* genotyping obtained a sensitivity and specificity of 51% and 77% respectively compared with tissue genotyping with the same platform. Within the ITT populations, patients *T790M*-positive by plasma and randomized to treatment (n=179) obtained a median PFS of 8.2 months and a ORR 77% similar to obtained by tumor positive *T790M* population (median PFS 10.1 months and ORR 71%) (20).

However it is salient to mention that all these plasma genotyping validation studies, with the exception of the Oxnard's cohort (18), were done exclusively in those patients with tissue positive *T790M* status, so this could have biased the results reported with regards the value of plasma in capturing those tissue *T790M* false negative cases.

ctDNA *T790M* testing in NSCLC: the hidden pitfalls

In addition to Cobas, a multitude of other platforms have

reported validation reports for ctDNA *T790M* mutation detection in blood (Table 1). By using tissue genotyping as “reference”, digital and non-digital PCR platforms exhibit a consistent moderate sensitivity (51–81%) and specificity (58–79%). Albeit, prospective data is only available for a limited number of these studies, while others report data based on very small number of cases.

Due to the high positive predictive value of plasma of these platforms and the high rate of false negative cases, authors recommended that plasma genotyping should be used as a complementary tool for *T790M* testing. The true percentage of *T790M* false-positive cases detected by plasma genotyping is probably lower than reported because some of them are false negative cases of tumor genotyping. If plasma genotype is positive for *T790M* this result predicts excellent outcome but if you get a negative *T790M* plasma result guidelines recommend to perform a re-biopsy for tissue genotyping to discard a true false negative plasma genotyping and to rule out other mechanisms of resistance such as HER2, MET amplification or small cell lung cancer transformation (1,14).

Various degrees of validation exist for individual plasma genotyping assays and the need of using tissue-genotyping as the reference standard method for the majority of validated genotyping assays to date is still a matter of debate. On the one hand, *T790M* mutation might be as well underestimated in tissue biopsies due to spatial and temporal tumor heterogeneity (10) and on the other side new orthogonal NGS genotyping assays might be more sensitive (90–95%)

(Table 1) than the aforementioned tests and capture a broader spectrum of genetic features in blood (22,23).

The study published by Remon *et al.* (15), differs from the others because they prospectively evaluated osimertinib efficacy but using only ctDNA for *T790M*-mutation detection without a matched tumor tissue sample used as reference. As for the platform used, eTam-SeqTM, it is a deep sequencing assay that allows for more extensive multiplexing including hotspot regions and copy number variations with potential predictive roles for patient's outcome. They detected three different cases of concomitant mutations (*PI3KA*, *STK11* and *NRAS*) along with *T790M* mutation. Indeed, this NGS platform obtained high sensitivity rates (92–100%) in preliminary analytical validation (25); in line with other NGS test (22,23). The *T790M*-positive rate of 50% and the similar outcomes obtained with osimertinib (ORR 62.5% and 6 months-PFS 66.7%) are consistent with previous studies in the same patient population (17–20). However, consideration should be given to the small sample size of patients evaluated for outcome (n=16) calling into question the reproducibility of the results. Moreover, as results were focused solely on *T790M*-positive population, data about false negative, sensitivity or specificity cannot be extrapolated. Similar to other studies (19) in this cohort no correlation was found between RECIST response and the frequency of *T790M* allelic fraction (AF). One explanation could be the long interval of time between the blood test and the start of osimertinib (median time of 1.5 months). Indeed, turnaround time of plasma NGS assays is potentially slower than other PCR-based assays such as Cobas. Correlations between RECIST response with osimertinib and ctDNA predictors (*T790M* AF, EGFR activating mutation AF and ratio of *T790M* and EGFR activating mutation AF) were also evaluated, and a non-significant trend was observed for lower mutant AFs of *T790M* or EGFR sensitizing mutations. The overall burden of metastatic disease in a patient has been previously demonstrated to predict for increased plasma genotyping sensitivity (24). However, in Remon's study, no data about tumor volume was reported and no correlation was given between tumor burden and depth of response challenging the real significance of low AF detection in this plasma samples.

ctDNA *T790M* testing in NSCLC: looking ahead

Based on the concept that ctDNA *T790M*-positive detection is a good predictor of osimertinib treatment outcome it is reasonable to move forward designing prospective trials in which treatment decisions are based on plasma genomic

testing results. The APPLE trial (NCT02856893) is a phase II trial for EGFR-mutant TKI-naïve NSCLC patients to evaluate the best strategy of treatment sequencing with gefitinib and Osimertinib (26). All patients (n=159) will be randomized (1:1:1): arm A will receive osimertinib as first-line treatment until disease progression according to RECIST criteria; whereas arms B and C will explore the procedure to switching from gefitinib to osimertinib at disease progression. However, and of key interest, definition of progression varies between later arms: arm B will employ molecular progression, that is to say plasma ctDNA detection of *T790M*-positive mutation, while arm C will use conventional RECIST disease progression criteria (Figure 1). This trial is based on the use of liquid biopsy by Cobas EGFR Mutation Test V2 in front of classical radiological criteria as a tool for making treatment decisions. They hypothesize that anticipating osimertinib use to RECIST progression by using plasma *T790M* testing will impact positively in patients' outcome (26).

However, based on the recent outstanding results reported with osimertinib in the first line setting (AURA phase 1 trial) with median PFS of 22 months (27), the role of liquid biopsy for *T790M* detection is definitively uncertain. The use of other blood-platforms that allows for a more extensive multiplex analysis, such as NGS, might be more appropriate to identify other new mechanisms of resistance to osimertinib.

Conclusions

Current guidelines use ctDNA as a complementary tool to detect *T790M* as a mechanism of resistance at EGFR-TKIs progression. Multiple new platforms with different technical features have recently entered in the landscape of NSCLC and have obtained different results in terms of sensitivity and specificity being Cobas EGFR mutation testing platform v2, the first blood test approved in the clinic for guiding treatment decisions in NSCLC.

Remon *et al.* have reported the first evidence and “proof of principle” of the potential of liquid biopsies in the real-world setting, by electing personalized-treatment strategies based solely on plasma ctDNA instead of the standard tissue-genotyping. However, and for the time being, it is advised to carefully validate beforehand all these platforms against the ‘standard’ albeit ‘non-optimal’ tissue-genotyping in the setting of prospective clinical trials before their routine use in clinical practice.

Prospective clinical trials comparing higher sensitive platforms such as NGS for *T790M* detection in tissue,

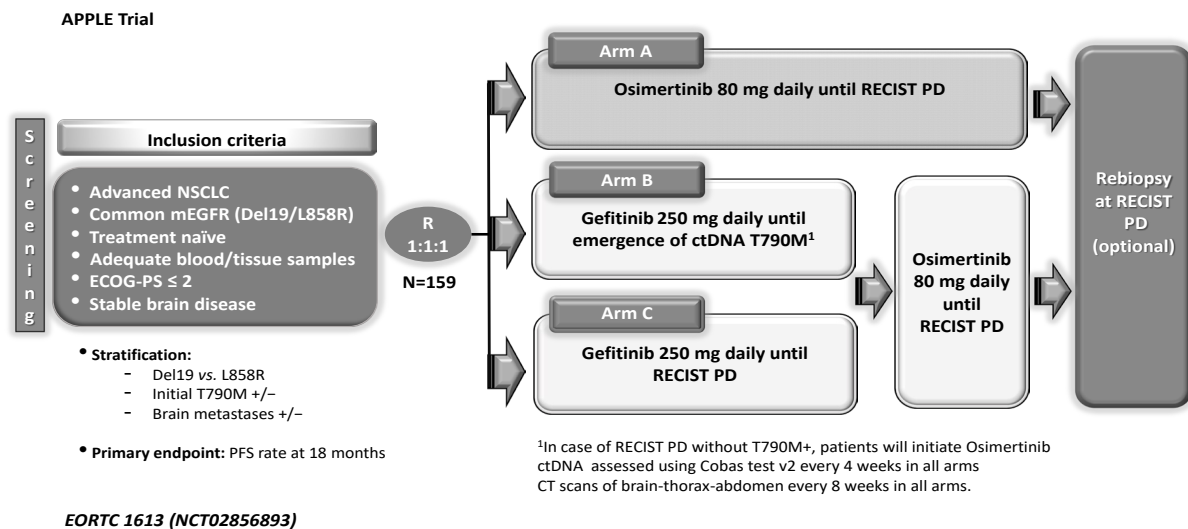


Figure 1 Flowchart of the randomized, multicentre, 3-arm, phase II, APPLE Trial [AZD9291 (Osimertinib) Treatment on Positive PLasma T790M in EGFR-mutant NSCLC Patients (APPLE) trial (EORTC 1613-NCT02856893)]. RECIST, response evaluation criteria in solid tumors; NSCLC, non-small-cell lung cancer; EGFR, epidermal growth factor receptor; ctDNA, circulating-free tumor DNA; PD, progressive disease; ECOG-PS, Eastern Cooperative Oncology Group-Performance Status; Rebiopsy, make a second or subsequent biopsy.

plasma or other biological liquids at disease progression are needed to establish the real role and future of ctDNA in daily clinical practice.

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aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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