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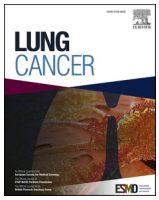

Research Paper

Combined analysis of circulating tumor DNA and tumor tissue to overcome osimertinib resistance (OSIRIS); the second line osimertinib cohort

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ABSTRACT

Introduction: Osimertinib resistance inevitably occurs in EGFR mutated NSCLC. We aimed to identify resistance mechanisms (RM) using paired plasma and tumor samples in patients that progressed on 2nd/3rd line osimertinib.

Methods: From 09 – 2019 to 02 – 2021, 51 patients were enrolled. Plasma sequencing used AVENIO Expanded Panel (research use only), tumor biopsies underwent DNA and RNA sequencing and histological evaluation. Sequencing was regarded successful when the driver mutation was confirmed with a variant allele frequency of ≥0.10%. Concordance between modalities was calculated for the driver mutation and RMs covered in both modalities. The Molecular Tumor Board formulated a treatment advice.

Results: The driver mutation was detected in 42/51 plasma samples (82%) and in 50/51 tumor samples (98%), concordance rate was 80%. In 41/51 (80%) patients ≥1 RM was identified. Thirty-two RMs covered in both modalities were found in plasma (61.5%), 39 in tumor (75%), nineteen in both. RM concordance rate was 36.5%.

Conclusion: Combined analysis of plasma and tumor samples post 2nd/3rd line osimertinib identifies additional RMs regardless of the comparative approach used. Plasma sequencing identified 61.5% of RMs, tumor analysis identified 75%. Combined, they provide a superior overview of osimertinib resistance, enabling more tailored treatment options.

1. Introduction

The development of targeted therapy for patients with epidermal growth factor receptor (EGFR) mutated non-small cell lung cancer (NSCLC) has drastically improved their treatment outcomes.

Due to therapeutic superiority when compared to chemotherapy, EGFR tyrosine kinase inhibitors (TKIs) have become the preferred treatment for patients with advanced EGFR mutated (EGFRm+) NSCLC [1,2]. Prior research has shown that the third generation EGFR TKI osimertinib outperforms earlier generation EGFR TKIs in untreated EGFR-mutated NSCLC [3,4] and outperforms chemotherapy in patients previously treated with earlier generation EGFR TKIs when EGFR T790M emerges [5].

However, as with earlier generation EGFR TKIs, progression inevitably occurs in the vast majority of patients treated with osimertinib. A variety of acquired osimertinib associated resistance mechanisms (RMs) have been described [6–9]. Among these are secondary (or, when T790M is present, tertiary) mutations in EGFR, amplification of MET or

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ERBB2, aberrations in the MAPK-pathway, and transformation to small-cell or squamous cell lung cancer. These RMs are not mutually exclusive and may coexist in the same patient or even in the same tumor lesion [10]. The genetic escape mechanisms may not only coincide spatially, but also change over time [11]. Proper clinical management dictates that these RMs should be investigated as these may be overcome with targeted treatment [8,12–14].

At the time of initiation of this study, there were no published data on prospective research focusing on acquired resistance to osimertinib in matched plasma and tissue biopsies.

In this study, we prospectively assessed RMs in EGFRm+ NSCLC patients with second or third line osimertinib failure. Plasma as well as a tissue biopsy were analyzed to identify potential RMs. The primary aim of the study was to provide the treating physician with a complete osimertinib resistance analysis and a recommendation for subsequent treatment, formulated by a multidisciplinary molecular tumor board (MTB).

2. Methods

2.1. Study design

In this single-center prospective study, we aimed to enroll 50 patients with metastatic NSCLC, characterized by a sensitizing EGFR mutation, who progressed after second line therapy with osimertinib 80mg once daily. In order to be eligible to participate in this study, patients had to meet all of the following criteria (Table S1): histologically confirmed metastatic NSCLC, characterized by a sensitizing EGFR mutation, progressive disease as assessed by the treating physician during osimertinib monotherapy, eligible for subsequent treatment, and be willing to undergo a tumor biopsy and provide a blood sample for ctDNA analysis.

All patients provided written informed consent. The study protocol, informed consent form and the information letter were approved by the Netherlands Cancer Institute's Institutional Review Board (IRB), as well as subsequent amendments. The study was registered under ClinicalTrials.gov Identifier NCT04737382. The study was funded by AstraZeneca and supported by an institutional grant of the Dutch Cancer Society and the Dutch Ministry of Health, Welfare and Sport. The study was performed in accordance with the Declaration of Helsinki.

After consent, patients were scheduled for a vena puncture and tumor biopsy to identify the mechanism(s) of osimertinib resistance. Results were discussed in a weekly organized Molecular Tumor Board, where a recommendation for the next line of treatment was formulated. Responses to the next line of therapy were monitored by the investigators.

Patients unable to undergo both a tissue biopsy and a plasma biopsy were excluded from the study and replaced. If more than a month had passed between the plasma biopsy and the tissue biopsy patients were also excluded and replaced.

2.2. Study outcomes

The primary objectives were to provide the treating physicians with access to complete osimertinib resistance analysis for their patients included in this study and to discuss these results in an MTB meeting to provide a recommendation for subsequent treatment.

Secondary objectives were evaluation of the success rate of molecular profiling on tumor tissue and ctDNA, defined as detection of the driver mutation, concordance between osimertinib resistance in plasma using ctDNA and in tumor tissue using a descriptive analysis, and concordance between recommended and actually provided subsequent treatment. Concordance between osimertinib RMs found in plasma and tumor tissue was only calculated for aberrations covered by both panels and for patients in whom both biosources showed the driver mutation.

Exploratory objectives included monitoring of clinical outcomes of the subsequent line of treatment in terms of progression free survival (PFS) and overall survival (OS).

PFS was defined as the time from initiation of first post-osimertinib treatment until progression on imaging, clinical deterioration or death, whichever occurred first. OS was defined as the time from initiation of first post-osimertinib treatment until death.

2.3. Tissue and plasma analysis of resistance mechanisms

For all patients, a histological biopsy of a tumor lesion or cytological analysis of malignant pleural effusion or a lymph node, as well as a blood sample for ctDNA analysis, were collected. All tissue biopsy analyses were performed using formalin-fixed, paraffin-embedded (FFPE) tissue or cell blocks from cytology specimens. For whole genome sequencing (WGS), samples were handled according to the protocol of the WIDE study [15].

All biopsies were morphologically assessed for histological transformation to squamous or small-cell phenotype, confirmed by immunohistochemistry (IHC).

If the tumor cell percentage (TCP) was sufficient (≥10%), DNA and RNA next generation sequencing (NGS) and/or whole genome sequencing were performed.

2.4. IHC

HER2 immunohistochemistry was performed using (4B5) Rabbit Monoclonal Primary Antibody by Roche. Intensity was divided in four categories: negative, low, moderate and strong intensity.

PD-L1 IHC was performed using PD-L1 IHC 22C3 LDT assay. Expression was characterized according to a tumor proportion score (TPS), resulting in a categorical distribution: negative (<1%), weakly positive (1–49%) and strongly positive (≥50%). These categories were then noted as a percentage of all tumor cells.

2.5. DISH and FISH

HER2 DISH was performed using the VENTANA HER2 Dual ISH DNA Probe Cocktail (Roche, Basel, Switzerland).

MET (F/D)ISH was performed using a centromere probe and a Dual Color MET-Cen7 probe (Roche Diagnostics). The average of the raw counts was reported as: MET/ CEP = average MET spots counts per cell/ average CEP spots counts per cell. If MET clouds were present these were reported as small (by eye estimated as less than 10 spots) or big clouds (by eye estimated as more than 10 spots).

2.6. DNA and RNA analysis

For tissue, DNA and RNA were isolated using Qiagen AllPrep DNA/ RNA FFPE kit (Qiagen N.V., Venlo, The Netherlands), according to manufacturer's instructions. DNA was stored at −20°C, RNA at −80°C.

DNA NGS was performed using Illumina Ampliseq™ Cancer Hotspot Panel v2-SOCv1 (Illumina, Inc., San Diego, CA, USA), RNA NGS used Archer FusionPlex Lung version 1 (IDTDNA, previously Invitae and Archer Dx, Boulder, CO, USA). Sequencing was performed using the Illumina MiSeq platform. WGS was performed at Hartwig Medical Foundation (Amsterdam, The Netherlands) on Illumina NovaSeq6000 platforms, using standard procedures on frozen material described previously [16,17].

For plasma biopsies, 20 ml of K2-EDTA was collected and processed within 3 hours using a two-step centrifugation protocol (20 min. at 380xg to isolate the platelets after which the supernatant was centrifuged for 10 min at 16,000xg to obtain cell free plasma). DNA isolation was performed using Qiagen Symphony DSP Circulating DNA kit, according to manufacturer's instructions. All samples were stored at −80° until analysis. Library prep was performed using the AVENIO ctDNA expanded kit (research use only (RUO)) according to the guidelines from the manufacturer. Sequencing was performed using the

NextSeq HS550. Analysis was done through Roche AVENIO ctDNA analysis software. For ctDNA analysis, variants were reported when variant allele frequency (VAF) was at least 0.10%. For copy number variation (CNV) analysis, a CNV score of ≥5 combined with a genomic region of <20% of the affected chromosome was required for amplification calling, with a genomic region <5% being interpreted as a probable amplification and genomic region ≥5% but <20% as possible amplification. For clarity, all 'probable' and 'possible' amplifications were counted as amplifications in this paper. ctDNA was analyzed for all pathogenic variants, and all variants were analyzed and classified by a clinical molecular scientist.

All single nucleotide variants (SNVs), fusions and CNV calls included in utilized panels are included in the Supplementary Methods.

2.7. Molecular tumor board

Results from tumor and plasma biopsy analyses were discussed in a weekly organized MTB, a multidisciplinary panel consisting of thoracic oncologists, specialists in laboratory medicine, pathologists and clinical molecular scientists. At least the following aspects were discussed: 1. RM identified 2. Targetability 3. Preferred treatment. Recommendations were based upon both current literature as well as expert opinion. Targetable RMs were BRAF V600E mutation, BRAF fusion, EGFR C797X, G724S or L718Q alone or in trans with T790M, MET amplification and ALK/ROS1/RET fusions.

The treating physician was then informed of the MTB recommendation. Turnaround time was defined as number of days between date of progression on osimertinib and discussion of results in MTB. Time between tissue biopsy and MTB recommendation was also recorded.

2.8. Follow-up

Compliance to the MTB recommendation was monitored, as well as OS and PFS following RECIST 1.1 or as assessed by treating physician, to the subsequent line of therapy. Assessment of OS, PFS and compliance to MTB recommendations was done via medical record review. Frequency of response evaluations were left to usual clinical care, unless patients were included in a clinical trial in which follow-up was defined.

2.9. Analysis

Statistical analysis was performed using IBM SPSS 27 (IBM, Armonk, NY, USA). For the exploratory survival data analysis, the Kaplan-Meier method with a log-rank test was used. A probability value of $p<0.05$ was considered to be significant. Survival data were compared between patient groups based on treatment after osimertinib (chemotherapy vs. targeted therapy). For patients receiving targeted therapy, data were stratified for number of RMs (one vs. multiple) in a post-hoc analysis. Survival data from patients who were still alive at time of analysis were censored by last recorded date that the patient was known to be alive. Hazard ratios were calculated using cox-regression analysis.

3. Results

Between September 2019 and February 2021, sixty patients were screened for inclusion and signed the informed consent document. Fifty-one patients were included and analyzed. Reasons for exclusion are summarized in Supplementary Flowchart S1. Patient characteristics at time of inclusion are provided in Table 1.

All patients were treated with at least one earlier generation EGFR TKI and 19 patients were also treated with platinum doublet chemotherapy prior to treatment with osimertinib.

3.1. TKI treatment prior to treatment with osimertinib

Forty-five patients received first generation EGFR TKI and six patients second generation EGFR TKI. Two patients were treated with two EGFR TKIs before being treated with osimertinib (erlotinib followed by rociletinib [n=1], gefitinib followed by erlotinib [n=1]). Median treatment duration with 1st/2nd generation EGFR TKI(s) was 13.6 months (95% confidence interval [95%CI]: 10.7–16.5 months). 47/51 (92%) of patients were T790M positive prior to initiation of treatment with osimertinib. The remaining four patients switched to osimertinib due to intracranial disease progression without confirmation of T790M status.

3.2. Osimertinib treatment

The median time to progression (as assessed by the treating physician) on osimertinib was 14.9 months (95%CI: 7.1–22.6 months). The

Table 1
Patient characteristics at time of inclusion for all patients and per treatment category post osimertinib.

Core characteristics	All patients	Chemotherapy	Targeted therapy	TT – one RM	TT – multiple RMs	Other/no therapy
Patients – n	51	19 (37 %)	17 (33 %)	7 (14 %)	10 (20 %)	15 (29 %)
Median age (range) – years	65 (37–85)	65 (39–82)	67 (37–78)	66 (54–78)	63 (37–73)	66 (47–85)
Female (%) – n	41 (80 %)	17 (89 %)	14 (82 %)	5 (71 %)	9 (90 %)	10 (67 %)
WHO PS						
WHO 0 – 1	47 (92 %)	18 (95 %)	15 (88 %)	6 (86 %)	9 (90 %)	14 (93 %)
WHO 2 – 3	4 (8 %)	1 (5 %)	2 (12 %)	1 (14 %)	1 (10 %)	1 (7 %)
Smoking status (%) – n						
Never smoker	29 (57 %)	12 (63 %)	9 (53 %)	4 (57 %)	5 (50 %)	8 (53 %)
Former smoker	21 (41 %)	7 (37 %)	7 (41 %)	3 (43 %)	4 (40 %)	7 (47 %)
Current smoker	1 (2 %)	0 (0 %)	1 (6 %)	0 (0 %)	1 (10 %)	0 (0 %)
Driver mutation (%) – n						
EGFR exon19del(/ins)	37 (73 %)	16 (84 %)	10 (59 %)	3 (43 %)	7 (70 %)	11 (73 %)
EGFR exon21mut	13 (25 %)	3 (16 %)	7 (41 %)	4 (57 %)	3 (30 %)	3 (20 %)
EGFR exon18mut	1 (2 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	1 (7 %)
Prior EGFR TKI treatment (%) – n						
Erlotinib	34 (67 %)	16 (84 %)	9 (53 %)	5 (71 %)	4 (40 %)	9 (18 %)
Gefitinib	9 (18 %)	3 (16 %)	4 (24 %)	2 (29 %)	2 (20 %)	2 (4 %)
Afatinib	6 (12 %)	0 (0 %)	4 (24 %)	0 (0 %)	4 (40 %)	2 (4 %)
Erlotinib, rociletinib	1 (2 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	1 (2 %)
Gefitinib, erlotinib	1 (2 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	1 (2 %)
Median first (and second) line EGFR TKI treatment duration (range) – months	13 (1–52)	12 (1–34)	13 (1–52)	16 (1–52)	13 (7–26)	18 (5–46)
Median osimertinib treatment duration (range) – months	15 (2–63)	13 (2–52)	9 (2–53)	29 (4–53)	6 (2–40)	20 (5–63)

pattern of progression was as follows: five patients had both intracranial and extracranial progression and 46 patients had extracranial progression only.

3.3. Tumor samples

Tumor samples were obtained from lung [n=21], any lymph node [n=10], liver [n=6], bone [n=7], muscle [n=3], and left adrenal gland [n=2]. In two patients, pleural effusion was collected. The complication rate of tumor biopsy procedures, defined as complications requiring further treatment, was 6%, with two pneumothoraxes requiring a pleural catheter placement and one patient with hemorrhage from a thoracic vertebra caused by the support needle requiring oral analgesics. Hospital stay was respectively two and five nights for the patients with a pneumothorax.

3.4. Tissue and plasma analysis of resistance mechanisms

Ten biopsies underwent NGS (Ampliseq) only, 37 biopsies underwent NGS as well as WGS, and four biopsies underwent WGS only. Molecular DNA analysis on tumor tissue was successful, defined as detection of at least the primary driver, in 50 patients (98%).

After a median of 13 days after determination of progressive disease (range: 0–74 days), all patients underwent a venipuncture for plasma sequencing. The driver was detected in 42 plasma samples (84%).

For 41 patients, matched samples with successful molecular profiling in both biosources were available revealing a concordance rate for primary driver identification of 80.4% (Supplementary Results).

In the total population, we detected at least one RM in 41 patients in plasma and/or tumor biopsy, of which 34 patients belonged to the group of 41 patients with successful analysis of both plasma and tumor. In 28 patients (54.9%), at least one RM was targetable. Fig. 1 visualizes the distribution of RM and the concordance between tissue and plasma.

In 50 successful tumor biopsies, we detected 32 amplifications in 26 patients, 5 fusions in 5 patients, 22 single nucleotide variants (SNV) in 18 patients and one case of EGFR vIII. All RMs detected in tumor biopsies were covered in the standard-of-care (SOC) NGS panel. WGS did not identify additional RMs that the SOC NGS panel missed. Plasma biopsies, successful in 42 patients, detected 9 amplifications in 9 patients, two fusions in two patients and 21 SNVs in 16 patients.

3.5. Single nucleotide variants

For the 41 patients with successful analysis of plasma and tumor, 18 patients had at least one SNV as a potential RM in either or both biosources, with a total of 24 SNVs.

All SNVs were covered in the utilized panels for both tissue and plasma. Of the 24 detected SNVs, 22 were detected in plasma, seventeen in tumor, and fifteen in both, resulting in a concordance rate of 62.5%.

3.6. Fusions

Of these same 41 patients, seven harbored a fusion detected in plasma, tumor biopsy, or both. Three of these fusions were eligible for concordance calculations, two of which were detected in plasma only, one unique to tumor. The concordance rate for fusions therefore was 0%.

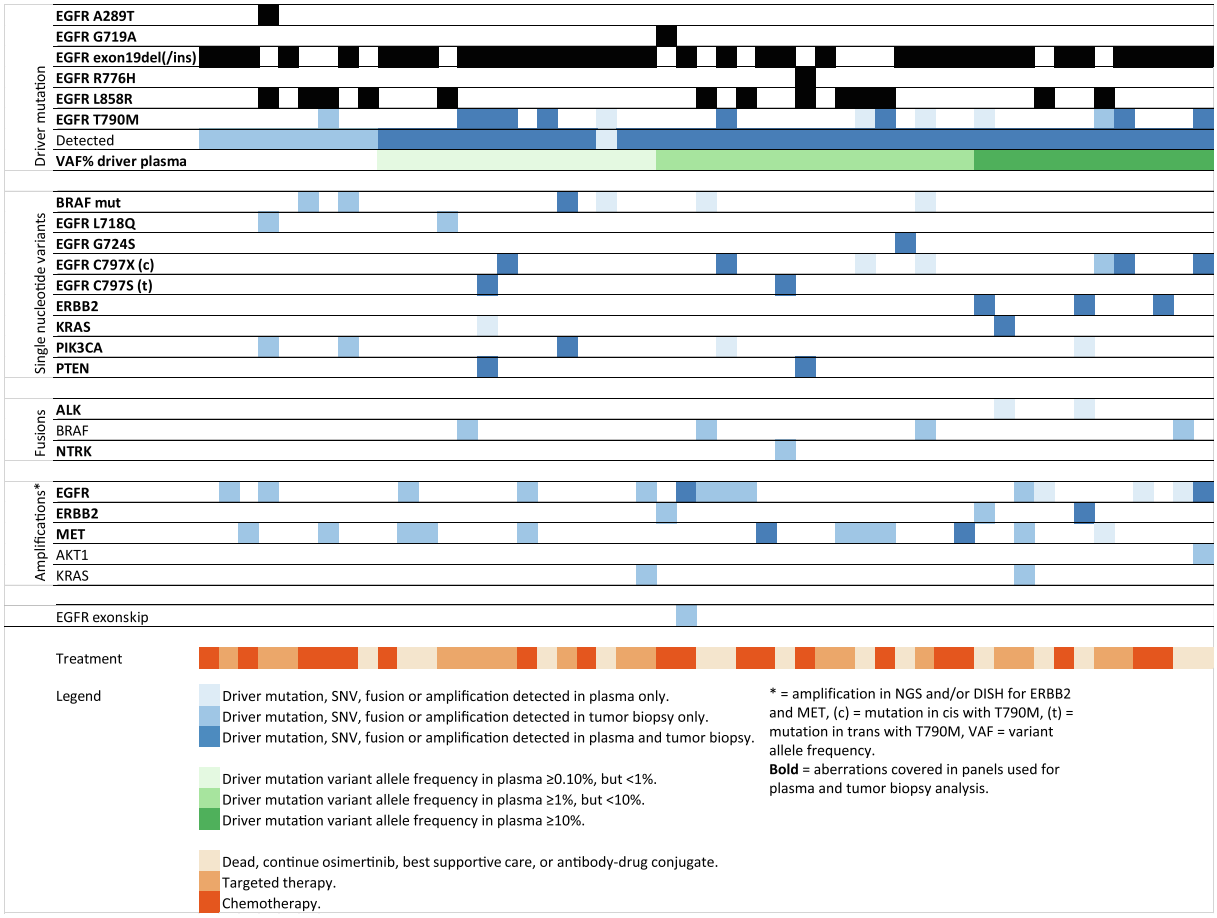


Fig. 1. Resistance mechanisms and therapy post osimertinib. Each column represents one study patient. Legend: * = deletion with/without insertion, ** = amplification in NGS and/or DISH for ERBB2 and MET, (c) = mutation in cis with T790M, (t) = mutation in trans with T790M, VAF = variant allele frequency. Bold = aberrations covered in panels used for plasma and tumor biopsy analysis.

3.7. Amplifications

For the 41 patients with successful analysis of both biosources, 22 patients had at least one amplification in plasma, tumor biopsy or both, with a total of 28 amplifications. Amplification of AKT1 [n=1] and KRAS [n=2] were only covered by the tissue panel. Therefore, 25 amplifications remained for concordance calculations. Nine were detected in plasma, 21 in tissue. Five amplifications were detected in both biosources, resulting in a concordance rate of 20% for amplifications.

3.8. EGFR vIII

EGFR vIII was not covered by the utilized plasma panel and was therefore not eligible for concordance calculations.

3.9. Total

In total, for the aforementioned 41 patients, 52 RMs were found across the categories mentioned above, with 32 (61.5%) detected in plasma, 39 (75%) in tissue and nineteen in plasma as well as tissue. Therefore, the total concordance rate was 36.5%.

3.10. MTB recommendation, treatment and outcome

All 51 included patients were discussed in an MTB meeting. Median turnaround time, from determining progressive disease while treated with osimertinib to formulation of MTB recommendation, was 40 days (IQR 32–56 days). Two patients deceased prior to MTB discussions due to rapid disease progression. Therefore, a treatment recommendation could be provided for 49 patients. Median time from tumor biopsy to MTB recommendation was 21.5 days. In 40 (82%) of these patients, the MTB treatment recommendation was executed. Reasons to deviate from MTB recommendation included slow progression, for which continuation of osimertinib was preferred over chemotherapy [n=2] or targeted therapy [n=1]; clinical deterioration [n=3]; and patient preference [n=2]. For one patient, surgical resection of the solitary growing lesion was recommended, but the patient was unable to undergo surgery. Therefore, a new line of systemic therapy was provided. Flowchart 1 provides a visual representation of categorized recommendation and adherence to the aforementioned recommendation.

Forty-eight patients received treatment after progression on osimertinib. Six patients continued osimertinib monotherapy due to slow or minimal progression of which five patients received additional local ablative therapy.

In 17 patients, a new line of targeted therapy based upon the elucidation of the presumed RM was initiated. Targeted treatment consisted of osimertinib in combination with: dabrafenib and trametinib for BRAF V600E mutation [n=3]; trametinib alone for MRKN-BRAF fusion [n=1]; gefitinib [n=1] or erlotinib [n=1] for EGFR C797S in trans; afatinib for EGFR G724S [n=1] or EGFR L718Q [n=1]; crizotinib for MET amplification [n=8] or ALK-fusion [n=1].

One of the aforementioned eight patients treated for MET amplification was treated with osimertinib and crizotinib after false positive MET amplification in plasma. This was due to AVENIO's automatically generated report, which called a high copy number variation of MET and EGFR. Upon closer inspection, the two amplicons were close to one another and, together, comprised more than 20% of the entire chromosome, suggesting polysomy of chromosome 7 rather than amplification of MET. At that time, the patient had already initiated the combination treatment. Interestingly, treatment led to a partial response lasting for a little over nine months. Two patients had a targetable RM, but were treated with chemotherapy based on the physician's choice. One of these patients had a TRIM24-BRAF fusion, the other had a MET amplification. In the latter case, due to clinical deterioration chemotherapy was initiated before the MET amplification result was known.

In 19 patients, treatment with chemotherapy was initiated. Chemotherapy consisted of: carboplatin combined with paclitaxel, bevacizumab and atezolizumab [n=2], carboplatin and pemetrexed [n=10], or paclitaxel and bevacizumab [n=7]. Additionally, six patients were treated with trastuzumab emtansine (T-DM1) for HER2 overexpression or amplification.

4. Survival analysis

4.1. Progression free survival

WHO Performance score for patients treated with chemotherapy and those treated with targeted treatment were similar (Table 1).

Patients that received targeted treatment after progression on osimertinib had a median PFS of 3.3 months (95%CI: 0.0–9.8 months), whereas patients treated with chemotherapy had a median PFS of 5.4 months (95%CI: 3.8–6.9 months), which was not significantly different (p-value = 0.91). Hazard ratio for disease progression or death in patients who received targeted therapy versus patients who received chemotherapy was 1.04 (95%CI: 0.53–2.04) (Fig. 2, Supplementary Tables S3 and S4). The patients treated with MET-directed therapies [n=8] had a median PFS of 7.1 months (95%CI: 0.0–15.4 months). Remaining subgroups performed worse (Supplementary Table S5).

4.2. Overall survival

Median OS for patients treated with targeted therapy was 15.8 months (95%CI: 12.6–19.1 months) vs 9.5 months (95%CI: 2.6–16.4 months) for those treated with chemotherapy, p-value = 0.35, hazard ratio for death in patients who received targeted therapy versus patients who received chemotherapy was 0.72 (95%CI: 0.36–1.44) (Fig. 3, Supplementary Tables S3 and S6).

Targeted therapy with one resistance mechanism versus multiple resistance mechanisms – PFS and OS

In a post-hoc exploratory analysis, survival data from patients with one RM and treated with targeted therapy were compared to the survival data of patients with more than one RM. Patients with one RM had longer PFS and OS (Supplementary Figs. S1 and S2).

5. Discussion

This report provides the first prospective analysis of RMs in matched plasma and tumor biopsy utilized for treatment advice in patients treated with second or third line osimertinib. Here, we show that in a dedicated cancer center it is feasible to obtain full molecular analysis of tumor and plasma biopsies and subsequent MTB recommendation with a clinically acceptable median turnaround time of 21.5 days from biopsy to treatment recommendation. However, it may be hypothesized that this turnaround time might be different in other health care settings.

The feasibility is further reflected by the fact that only three of 51 included patients were not able to receive subsequent anticancer treatment due to clinical deterioration. The most important findings of our study are that both tumor tissue as well as circulating tumor DNA are required to detect the mechanisms of osimertinib resistance and that prescreening with either plasma or tissue alone may be insufficient.

Since the initiation of this study, plasma ctDNA analysis has been marked as a complementary tool in the most recent IASLC consensus statement [18]. The present study supports this view, as 11 of 49 plasma biopsies showed additional RMs not detected in the tissue sample of the same patient. However, where IASLC recommends considering a 'plasma first' approach as standard of care, our data favors synchronous analysis of both tissue and plasma, with analysis of tumor biopsies yielding unique genomic information that could not be replicated in the plasma biopsies generated from the same patient in 17 of 49 patients. As the primary EGFR mutation could be detected in plasma for 11 of these patients, the discrepancy between tumor and plasma biopsies cannot be attributed to non-shedding tumors only. As it takes approximately 10

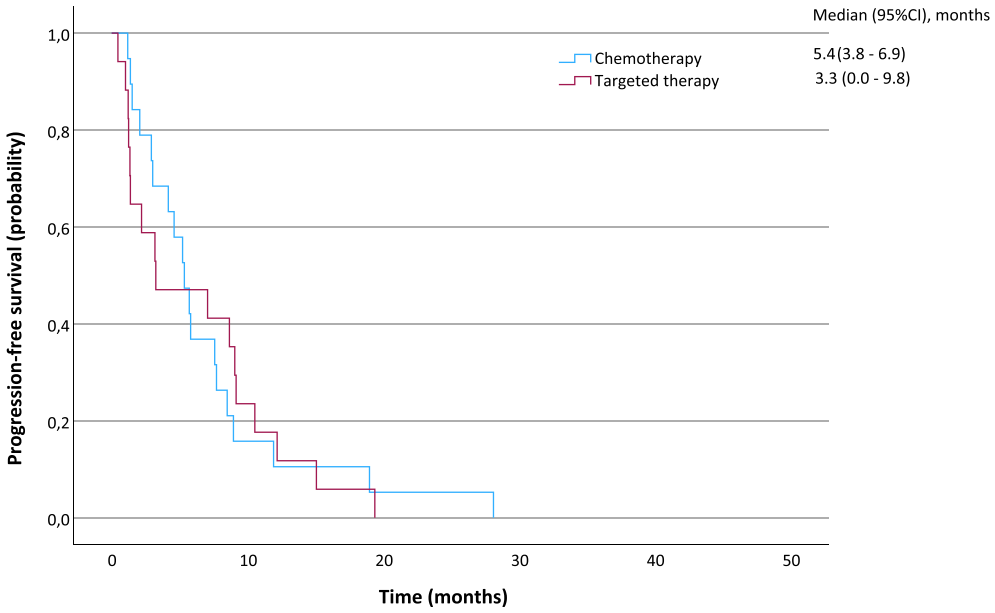


Fig. 2. Kaplan Meier curves for PFS in patients who received chemotherapy versus those who received targeted therapy.

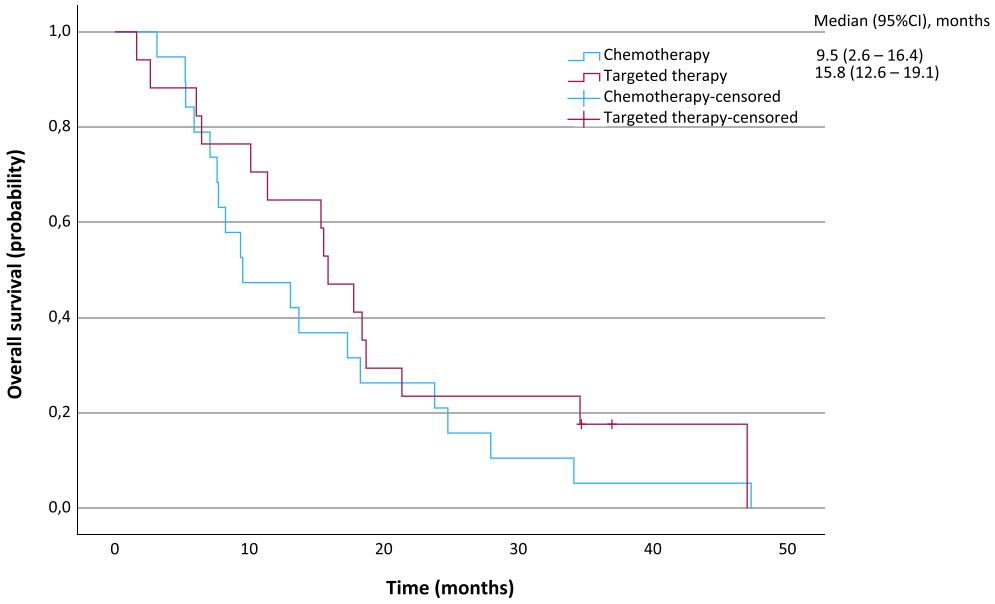


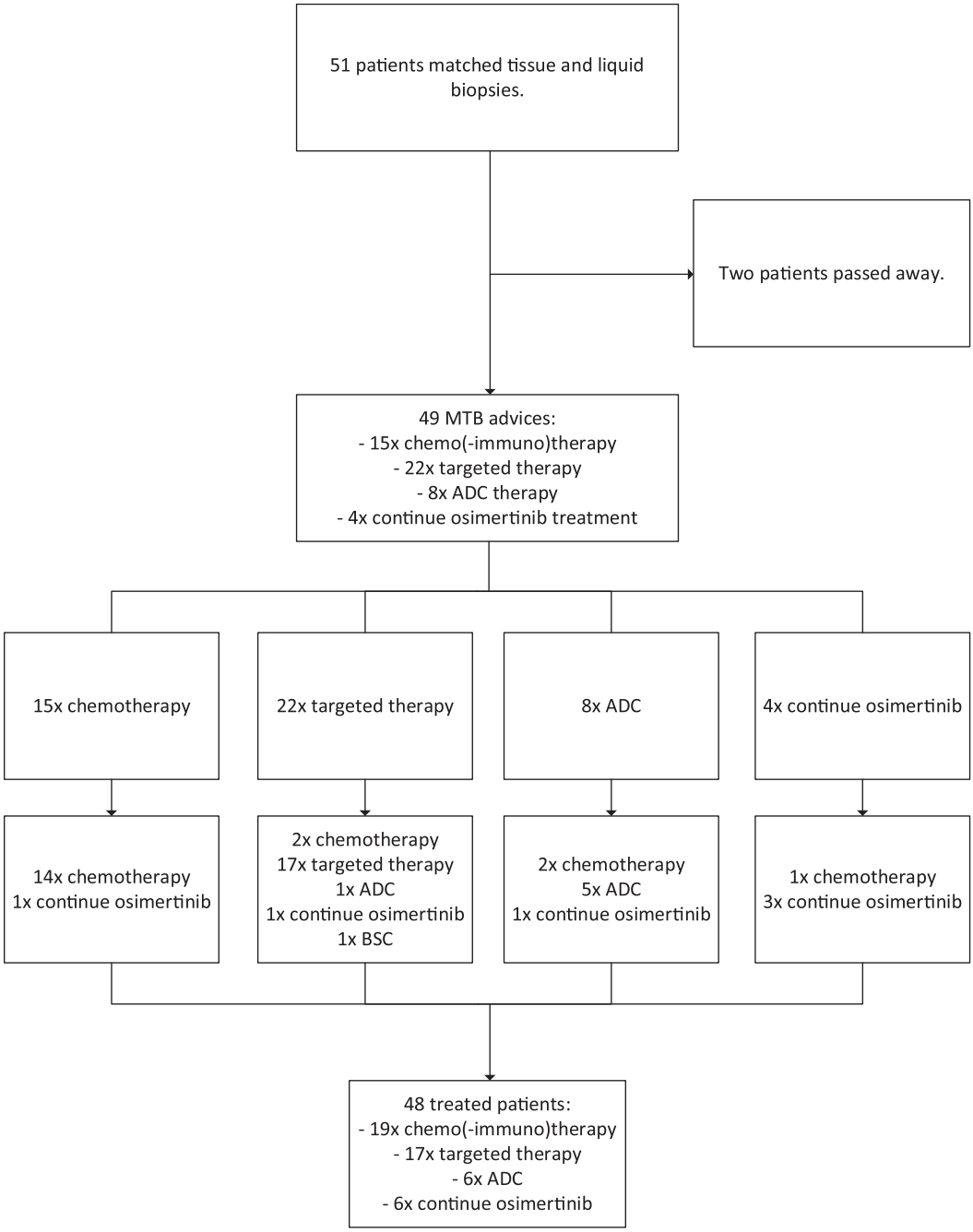
Fig. 3. Kaplan Meier curves for OS in patients who received chemotherapy versus those who received targeted therapy.

cm^3 of tumor to yield ctDNA fragments at about 0.1% [19], a sizable osimertinib resistant clone is required to detect RMs in plasma. However, in a sizable group of patients, we also found RMs in plasma but not in tissue, which is in line with the findings reported by Jee J, et al, where additional genomic alterations in ctDNA were found in 25% of patients that could not be detected in tumor tissue. These ctDNA-only alterations disproportionately featured subclonal drivers of resistance [20].

In recent years, tumor fraction assessment as a measure of the plasma sample's suitability for plasma sequencing has been suggested [21]. Unfortunately, this is not possible with AVENIO expanded panel (RUO), as this is a targeted panel and tumor fraction assessment requires shallow WGS. However, considering that the VAF is a representation of the tumor fraction [22], it is still possible to use the EGFR driver mutation VAF as a measure of the quality of the plasma sample. Although not powered for such analyses, the results show RM identification in plasma is more successful in samples with a high (more than 10%) EGFR driver mutation VAF. Consequently, caution is warranted when interpreting a negative RM analysis in plasma where the EGFR driver mutation is not detected or present with a low VAF, especially below 1%. This patient group highlights the importance of combining plasma and tissue analysis in the setting of osimertinib resistance.

In addition, some patients may develop resistance to osimertinib through small-cell transformation. While RB1 loss and TP53 mutations may be identified in plasma and highlight an enhanced risk of small-cell transformation [23,24], in itself these are insufficient evidence for this transformation as these can be found in absence of histological transformation. Also, the possibility of resistance to osimertinib through squamous cell carcinoma transition underlines the importance for morphological assessment in the setting of osimertinib failure [25]. Histological transformation to small cell lung cancer or squamous cell carcinoma was not observed in the patients included in this cohort.

Additionally, panels used for tissue and plasma sequencing differ in



Flowchart 1. MTB recommendation and treatment post-osimertinib.

their coverage for mutations, fusions and copy number variations. For example, KRAS amplifications (detected once through Ampliseq on tissue) and BRAF fusions (detected four times through Archer FusionPlex on tissue) are not covered in AVENIO's expanded panel (RUO). To some extent, this complicates analysis of concordance between tissue and plasma sequencing.

Furthermore, tumors may harbor primary resistance mechanisms, prior to any treatment. In our patients, we were unable to (re)analyze baseline biopsies to differentiate between resistance mechanisms that emerge during osimertinib treatment and those that were present prior to osimertinib treatment.

Progression-free survival for osimertinib treatment in this cohort (14.9 months, 95%CI 7.1–22.3 months) was similar to that described in AURA3 (10.1 months, 95%CI 8.3–12.3 months) [5], and in a recent real-world study, ASTRIS (11.1 months, 95%CI: 11–12 months) [26]. Overall survival was comparable to AURA3, but slightly longer than in ASTRIS: OSIRIS 30.0 months, 95%CI 26.3–33.8 months, AURA3 26.8 months, 95%CI 23.5–31.5 months [27], ASTRIS 22.8 months, 95%CI 21.6–23.8 months.

As the osimertinib resistance profile partially depends on the treatment duration [28,29], comparisons between reported resistance profiles is complicated. However, identifying MET amplification and secondary EGFR mutations as the most frequent RMs is in line with previous publications, both in clinical trials [30,31] as well as in real-world analyses [32]. Regardless, with similar treatment durations and resistance profiles, our study stands out by combining tissue and plasma analysis.

By sequencing both tumor tissue and circulating tumor DNA, it was possible to suggest a targeted treatment approach in a MTB meeting for 23 of the 51 included patients, underscoring the fact that molecular

guided therapy of osimertinib resistance is feasible.

While little is known about the most appropriate treatment following osimertinib resistance, the current standard of care is still platinum doublet chemotherapy.

Subsequent targeted therapies are currently under investigation [33–35], focusing predominantly on MET amplification, as this is the most common resistance mechanism to osimertinib. In addition, various antibody-drug conjugates are being researched. For example, a phase III trial comparing a HER3-directed antibody-drug conjugate (patritumab-deruxtecan, HER3-Dxd) to chemotherapy after osimertinib failure is underway [36]. In addition, trials investigating a TROP2-directed antibody-drug conjugate (Datopotamab-deruxtecan, Dato-Dxd) are ongoing, with promising preliminary results [37,38].

Patients treated with chemotherapy had similar response durations when compared to earlier studies in this disease setting [39–41]. Patients with osimertinib and MET-directed therapy achieved a median PFS of 7.1 months. Results for targeting other RMs were disappointing.

Studies on targeting MET amplification as resistance mechanism focused on combining osimertinib with crizotinib [12,42–44], savolitinib [35] or tepotinib [45]. The combination of osimertinib and crizotinib reported by Wang et al. rendered a median PFS of 6.2 months [44]. Sequist et al. report a median PFS of 5.4 months in patients treated with osimertinib and savolitinib after progressing on osimertinib monotherapy [46]. Mazieres et al. report that half of responders had a duration of treatment of at least six months, and median duration of response was not reached [45]. In the report of Sequist et al., the threshold for MET amplification in NGS was a gene copy number of at least 5, where Mazieres et al. defined MET amplification in NGS as a gene copy number of at least 2.3. This shows that various medical centers and research groups apply different thresholds for MET amplification, which warrants caution when it comes to comparing treatment outcomes.

The studies mentioned above, and the one reported here, all analyzed small patient groups and applied different MET directed treatment combinations.

Results of the exploratory post-hoc analysis comparing patient groups based on the number of RMs may show that durable responses can be obtained in patients with only a single RM. Recent reports show similar findings. Cho et al. [47] describe that none of the patients with EGFR- and/or MET-independent resistance mechanisms after osimertinib failure achieve a response to combined amivantamab (an EGFR-MET bispecific antibody) and lazertinib (a third generation EGFR TKI) treatment. Hartmaier et al. report a possible correlation between non-response to osimertinib and savolitinib treatment in those patients with EGFR C797S or RICTOR amplification in combination with the MET amplification. These findings should be interpreted with caution due to the number of patients and exploratory nature of the analyses, but do highlight a necessity to further explore this in prospective studies. Future trials exploring combination therapies after osimertinib may consider excluding patients harboring two or more RMs, or at least stratify for the number of RMs detected by combined analyses of tumor tissue and ctDNA.

The results of our study suggest that complete molecular profiling of osimertinib resistance with both tumor tissue and circulating tumor DNA allows to identify those patients that benefit the most from subsequent targeted treatment combinations. Additional research in larger cohorts is warranted to confirm these results, as well as focusing on resistance to first line osimertinib treatment, as the resistance pattern may differ from those treated with second or third line osimertinib.

6. Additional information

6.1. Authors' contributions

J.W.T. van der Wel: was involved in the methodology, coordinated the study, and primarily wrote the manuscript. M. Jebbink: was involved in the conceptualization of the study, temporarily coordinated the study, and helped write the manuscript. D. van den Broek: was involved in the conceptualization of the study, helped interpret data and helped write the manuscript. L.C. Steinbusch: acquired data and helped write the manuscript. W.S.M.E. Theelen: acquired data and helped write the manuscript. G. Ruiter: acquired data and helped write the manuscript. W. Buikhuisen: acquired data and helped write the manuscript. J.A. Burgers: acquired data and helped write the manuscript. P. Baas: acquired data and helped write the manuscript. M. Vermeulen: coordinated the project administration and helped write the manuscript. V. Van der Noort: Advised and helped with statistical analysis and helped write the manuscript. S.M.S. Hashemi: acquired data and helped write the manuscript. L.J.W. Bosch: helped interpret data and helped write the manuscript. K. Monkhorst: was involved in the conceptualization of the study, helped interpret data and helped write the manuscript. E.F. Smit: was involved in the conceptualization of the study, acquired data, helped interpret data and helped write the manuscript. M.C. Boelens: helped interpret data and helped write the manuscript. A.J. de Langen: was involved in the conceptualization of the study, was the principal investigator, acquired data, helped interpret data and helped write the manuscript.

7. Ethics approval and consent to participate

All patients provided written informed consent. The study protocol, informed consent form and the information letter were approved by the Netherlands Cancer Institute's Institutional Review Board (IRB), as well as subsequent amendments. The study was performed in accordance with the Declaration of Helsinki.

CRediT authorship contribution statement

J.W.T. van der Wel: Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Investigation, Formal analysis, Data curation. **M. Jebbink:** Writing – review & editing, Resources, Project administration, Conceptualization. **D. van den Broek:** Writing – review & editing, Resources, Conceptualization. **L.C. Steinbusch:** Writing – review & editing, Resources. **W.S.M.E. Theelen:** Writing – review & editing, Resources. **G. Ruiter:** Writing – review & editing, Resources. **W. Buikhuisen:** Writing – review & editing, Resources. **J.A. Burgers:** Writing – review & editing, Resources. **P. Baas:** Writing – review & editing, Resources. **M. Vermeulen:** Writing – review & editing, Resources, Project administration, Conceptualization. **V. van der Noort:** Writing – review & editing, Formal analysis. **S.M.S. Hashemi:** Writing – review & editing, Resources. **L.J.W. Bosch:** Writing – review & editing, Resources. **K. Monkhorst:** Writing – review & editing, Resources, Conceptualization. **E.F. Smit:** Writing – review & editing, Supervision, Resources, Conceptualization. **M.C. Boelens:** Writing – review & editing, Resources. **A.J. de Langen:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Baas reports a grant from BMS, unrestricted to the hospital, as well as consulting fees paid to the hospital by BMS, MSD, AMheart, payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events to the hospital from BMS. Burgers reports a grant from MSD as support for an investigator initiated study, payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events to the hospital from MSD, participation on a data safety monitoring board or advisory board with Roche, paid to the hospital, and receipt of free drugs for an investigator initiated study from MSD. Hashemi reports grants from AstraZeneca, Boehringer Ingelheim, BMS, Lilly, Janssen, GSK, MSD, Novartis, Roche and Takeda, all to the institution. De Langen reports grants from BMS, grants from

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lungcan.2024.107972.

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