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1 **Molecular mechanisms of acquired resistance to MET tyrosine kinase inhibitors**
2 **in patients with MET exon 14 mutant NSCLC**
3

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9

10 **Running Title:** Resistance mechanisms to MET tyrosine kinase inhibitors

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15

16 **Statement of significance :**

17

18 Genomic mechanisms of resistance to MET tyrosine kinase inhibitors where
19 identified in 20 patients experiencing disease progression to type I or type II MET
20 inhibitors using tumor and/or plasma next-generation sequencing. On-target (secondary
21 *MET* kinase domain mutations, *MET* amplification) and off-target genomic alterations
22 (ERBB family of receptor tyrosine kinase gene amplification, *MAPK* pathway gene

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1 amplification and *KRAS* mutations) were detected. Switching between type I and type II
2 MET inhibitors resulted in partial responses in two patients. The understanding of the
3 biological mechanisms of resistance to MET inhibitors may guide the development of
4 novel treatment strategies.
5
6 **Conflict of interest statement:**

7 G.R. Consultant/advisory board: Roche, Pfizer, Amgen.

8 J.C. Consultant: Soltego, Kymera

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10 K.P and S.R.F are employees and stockholders of Guardant Health

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1 Ignyta, LOXO Oncology, Eli Lilly pharmaceuticals, Araxes pharmaceuticals, SFJ
2 Pharmarceuticals, Voronoi, Daiichi Sankyo, Biocartis, Novartis, Takeda Oncology. Stock
3 Ownership: Gatekeeper Pharmaceuticals, LOXO Oncology. Research Funding: Astellas
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7 M.M.A: Consultant/advisory board: Bristol-Myers Squibb, AstraZeneca, Achilles,
8 AbbVie, Neon, Maverick, Nektar, Hegrui, Syndax, Gritstone. Institutional research
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10 M.B, L.S, B.R, G.C.L, YC.L, Y.L, G.L, T.N., M.M., D.V, R.U., C.P.P., A.A: Nothing to
11 disclose.

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1 **Abstract**

2 **Purpose**: Molecular mechanisms of acquired resistance to MET TKIs are poorly
3 understood. We aimed to characterize the genomic mechanisms of resistance to type I
4 and type II MET TKIs and their impact on sequential MET TKI therapy outcomes in
5 patients with metastatic *MET* exon 14 mutant NSCLC.

6 **Experimental Design:** Genomic alterations occurring at the time of progression on
7 MET TKIs were studied using plasma and tissue next-generation sequencing (NGS).

8 **Results:** A total of 20 patients had tissue or plasma available for analysis at the time of
9 acquired resistance to a MET TKI. Genomic alterations known or suspected to be
10 mechanisms of resistance were detected in 15 patients (75%). On-target acquired
11 mechanisms of resistance, including single and polyclonal MET kinase domain
12 mutations in codons H1094, G1163, L1195, D1228, Y1230, and high levels of
13 amplification of the *MET* exon 14 mutant allele, were observed in 7 patients (35%). A
14 number of off-target mechanisms of resistance were detected in 9 patients (45%),
15 including *KRAS* mutations and amplifications in *KRAS*, *EGFR*, *HER3*, and *BRAF*; one
16 case displayed both on- and off-target mechanisms of resistance. In two patients with
17 on-target resistant mutations, switching between type I and type II MET TKIs resulted in
18 second partial responses.

19 **Conclusions**: On-target secondary mutations and activation of bypass signaling drive
20 resistance to MET TKIs. A deeper understanding of these molecular mechanisms can
21 support the development of sequential or combinatorial therapeutic strategies to
22 overcome resistance.

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1 **Introduction**

2 *MET* exon 14 alterations occur in ~3% of non-small cell lung cancers (NSCLCs)
3 and predict for response to treatment with MET tyrosine kinase inhibitors (TKIs)(1,2).
4 Several MET TKIs are under clinical development for the treatment of patients with
5 advanced *MET* exon 14 mutant NSCLC. Type I MET TKIs, such as crizotinib,
6 capmatinib, tepotinib, and savolitinib bind to MET in its catalytically active conformation
7 where the aspartic acid-phenylalanine-glycine (DFG) motif projects into the ATP-binding
8 site (DFG-in)(3–7). In contrast, type II MET TKIs such as cabozantinib, merestinib, and
9 glesatinib, bind to MET in its inactive DFG-out conformation(8–10). Type I MET
10 inhibitors are further subclassified as type Ia (crizotinib) when the drug interacts with the
11 solvent front G1163 residue, and type Ib (capmatinib, tepotinib and savolitinib) when
12 drug binding to the kinase domain is independent from this interaction. Early reports
13 indicate that response rates with type I MET TKIs range from 32% to 55%, and the
14 median progression-free survival (PFS) with these drugs varies between 5 and 12
15 months, and is limited by the invariable emergence of acquired resistance to these
16 therapies (4,5,11–13).

17 The landscape of resistance mechanisms to MET TKIs in patients is not well
18 characterized. Acquired *MET* kinase domain mutations in residues D1228 and Y1230
19 confer resistance to type I MET TKIs *in vitro* by weakening chemical bonds between the
20 drug and the MET kinase domain (8,14). In addition, the solvent front G1163R mutation
21 confers resistance to crizotinib but not to type Ib MET inhibitors like tepotinib, savolitinib
22 or capmatinib *in vitro* (15). By contrast, resistance to type II MET inhibitors can occur by
23 mutations affecting residues L1195 and F1200 (9,15).

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Preclinical studies suggest that mutations in specific residues can cause resistance to drugs that bind in a similar fashion and could be potentially overcome by switching to a type of MET TKI with different mode of binding (8,9,16). Off-target mechanisms of resistance have also been described in patients treated with MET TKIs, including but not limited to wild-type *KRAS* amplification and activating *KRAS* mutations (17,18). Nevertheless, the relative frequency of on-target and off-target resistance mechanisms in patients is not well known.

In this study, using tissue and blood-based NGS we assessed the genomic mechanisms of acquired resistance in tumors from patients with advanced *MET* exon 14 mutant NSCLC treated with MET TKI.

Materials and methods

Patients and clinical outcomes

Patients with *MET* exon 14 altered NSCLC treated at the Dana-Farber Cancer Institute (DFCI) with available paired tumor biopsies and/or plasma samples at baseline and at the time of resistance to treatment with a MET TKI were identified. Patients were included in this study if they had achieved a confirmed or unconfirmed partial response or any degree of initial target lesion shrinkage followed by disease progression, or if they had experienced disease progression after 6 months of stable disease while on treatment with a MET TKI. Patients experiencing primary progression to their first MET TKI were not included. Progression-free survival and time to treatment discontinuation of MET TKIs were estimated using the Kaplan-Meier method and groups were

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compared using the log-rank test. All patients provided written consent to institutional review board-approved protocols at the Dana-Farber/Harvard Cancer Center (DF/HCC) allowing for chart review and genomic sequencing on tissue and plasma samples [DF/HCC protocols #02-180, #16-374 (NCT02920996) and #14-147 (NCT022790049)]. The study was conducted in accordance with the Declaration of Helsinki.

Tissue and plasma next-generation sequencing

Baseline and MET TKI-resistant samples were analyzed using targeted NGS with the DFCI OncoPanel platform, as previously described (19). For patients who received prior care outside of DFCI, Clinical Laboratory Improvement Amendments (CLIA)-approved NGS assays were retrieved from electronic medical records. When plasma samples were available, cell-free DNA (cfDNA) was extracted and analyzed using the Guardant360 panel from Guardant Health, as previously reported (20).

Bioinformatic Analysis

Targeted next generation sequencing (NGS) was performed using the validated OncoPanel assay (19,21) at the Dana-Farber Cancer Institute Center for Cancer Genome Discovery for 277 (OncoPanel version 1), 302 (version 2), or 447 (version 3) cancer-associated genes. Briefly, tumor DNA was prepared as previously published, hybridized to custom RNA bait sets (Agilent SureSelectTM, San Diego, CA) and sequenced using Illumina HiSeq 2500 with 2x100 paired-end reads. Sequence reads were aligned to reference sequence b37 edition from the Human Genome Reference Consortium, using bwa, and further processed Picard (version 1.90,

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http://broadinstitute.github.io/picard/) to remove duplicates and Genome Analysis Toolkit (GATK, version 1.6-5-g557da77) to perform localized realignment around indel sites (22). Single nucleotide variants were called using MuTect v1.1.46 and insertions and deletions were called using GATK Indelocator. Variants were filtered to remove potential germline variants as previously published and annotated using Oncotator (23). In order to remove additional germline noise, variants were excluded which were annotated as benign/likely benign in ClinVar or were present at a population maximum allele frequency of 0.1%, retaining variants in either case if they were annotated as confirmed somatic in at least two samples in COSMIC (24,25). Copy number variants and structural variants were called using the internally-developed algorithms RobustCNV and BreaKmer (26). For each gene, the absolute copy number was estimated based on the tumor purity (p) and the weighted average of segmented log2 ratios across the gene (l) using the formula: $ACN = (2^{(l+1)}-2(1-p))/p$.
Due to regulatory constraints, the files required to estimate ploidy were unavailable for analysis. Our analyses assume a diploid status for all tumors which we recognize could impact the absolute copy number in aneuploid tumors. Arm-level copy number changes were generated using an in-house algorithm specific for panel copy number segment files. Chromosome arms were classified as amplified or deleted if more than 70% of the covered portion of arm was altered.

MET molecular modelling methods

Molecular dynamics (MD) simulations were performed for savolitinib and glesatinib in wild type and H1094Y MET kinase domain. The co-crystal structure of savolitinib with

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1 MET (pdbcode: 6SDE) was used as initial structure for MD simulation. Missing side
2 chains and loops were built using Prime in schrodinger suite (release 2019-2)(27,28),
3 and MD simulation of 500ns was carried out using Desmond on GPU. Trajectories were
4 saved every 50ps for production runs. Default equilibration protocol prior to production
5 was applied, and the long range interaction cut-off was set to 10 Angstrom. 1800 frames
6 were uniformly sampled from MD trajectories of last 450ns for binding free energy
7 calculation using MM-GBSA algorithm (Schrodinger Prime MM-GBSA module)(29). MD
8 simulation for the H1094Y mutation was carried out with the same procedure and
9 settings. The mutation was introduced directly to the initial structure and energy
10 optimized prior to MD setup and simulations. The protocol applied to MD simulations for
11 glesatinib as well. Since the cocrystal structure for glesatinib with MET was not
12 available at the time of calculation, glesatinib was modeled based on one of the
13 cocrystal structures of a close analog (pdbcode: 3C1X). Similarly, missing side chains
14 and loops were modeled with Prime prior to MD simulations. All binding free energies
15 were calculated by Prime MM-GBSA with default settings.
16
17 *Antibodies and Compounds*
18 Antibodies against phospho-MET (Tyr1234) was purchased from Santa Cruz
19 Biotechnology; total-MET (D1C2) and anti-rabbit IgG-HRP from Cell Signaling
20 Technology; α−tubulin from Sigma Aldrich; and anti-mouse IgG-HRP from GE Life
21 Sciences. Savolitinib, crizotinib, cabozantinib, and glesatinib were purchased from
22 Selleckchem; merestinib from MedChem Express.
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1 *Expression vectors*

2 Full length human *MET*, transcript variant 2, cDNA (NM_000245.2) was amplified from a
3 banked tumor specimen with an unrelated genetic alteration. The amplicon was
4 subcloned into pDNR-dual (BD Biosciences) via the *HindIII* and *XHOI* restriction sites as
5 described previously (30). Exon 14 was deleted from the full length *MET* cDNA
6 construct using the USB® Change-IT™ Multiple Mutation Site Directed Mutagenesis Kit
7 and (5'phospho) forward mutagenic primer:
8 gctgaaaaagagaaagcaaattaaagatcagtttcctaattcatctcagaacg, along with the reverse
9 primer provided with the kit. The *MET* H1094Y mutation was introduced using the
10 QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and the
11 following mutagenic primers: Forward: 5'- agagggcattttggttgtgtatattatgggactttgttgg -3';
12 Reverse: 5'- ccaacaaagtcccataatatacacaaccaaaatgccctct -3'. All constructs were
13 confirmed by DNA sequencing. Constructs were shuttled into the retroviral expression
14 vector JP1540 using the BD Creator™ System (BD Biosciences).
15
16 *Cell lines*

17 HEK-293T cells were purchased from ATCC (in 2009), cultured in DMEM,
18 supplemented with 10% FBS, streptomycin and penicillin and authenticated using the
19 Promega GenePrint 10 System at the RTSF Genomics Core at Michigan State
20 University in August 2016. All cell lines used in the study tested negative for
21 mycoplasma as determined by the Mycoplasma Plus PCR Primer Set (Agilent).
22
23

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1 *Drug treatments and Western blotting*
2 For transient MET (WT or mutant) overexpression, 5 x 10^5 293T cells were transfected
3 with 1 µg DNA and 6 µL FuGENE® HD (Promega) in Opti-MEM® media (Gibco). Media
4 were replaced 16 hours post-transfection with complete DMEM. 72 hours after
5 transfection, cells were treated with inhibitors for 6 hours and subsequently lysed for
6 Western blotting. Cell lysis, Western blotting, and immunoblotting were done as
7 described previously (31). Blots were developed on Amersham Imager 600 (GE
8 Healthcare Life Sciences).
9
10
11 **Results**
12 *Patients*
13 Between April 2014 and June 2019, 114 patients with NSCLC harboring *MET*
14 exon 14 splicing alterations were identified, of which 71 (62%) had metastatic disease
15 (**supplementary Figure 1**). Of these, thirty-nine patients (55%) received at least one
16 MET TKI and were evaluable for treatment response. In total, 29 patients had
17 documented disease progression on a MET TKI, and in 20 cases, tissue and/or plasma
18 NGS was performed at the time of progression. For their best objective response,
19 thirteen patients experienced a partial response, five had stable disease lasting ≥ 6
20 months and two patients experienced stable disease ≤ 6 months with some degree of
21 tumor reduction.
22 Baseline clinical and molecular characteristics of patients included in genomic
23 profiling at progression are summarized in **supplementary Table 1**. Among the 20

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patients who underwent NGS analysis of pre- and post-treatment samples, 14 received one MET TKI, and 6 received sequentially both type I and II MET TKIs. NGS was performed after progression on both types of MET TKIs in five cases, and in one case, treatment with a second-line MET TKI was still ongoing at the time of study cut-off.

Genomic mechanisms of resistance to MET TKIs

Mechanisms of resistance conferred by on- and off-target genomic alterations were identified in 15 cases (75%). On-target mechanisms of resistance, including *MET* kinase domain mutations or acquired focal amplification of the *MET* exon 14 mutant allele, were found in 7 cases (35%). Acquired *KRAS* mutations or acquired amplification of wild-type *EGFR*, *KRAS*, *HER3*, and *BRAF*, as single or compound mechanisms of resistance, were detected in 9 cases (45%). One case displayed both on- and off-target resistance with a D1228N *MET* kinase domain mutation as well as amplification of *EGFR* and *HER3.* In 5 cases (25%), the genomic mechanism of resistance to MET TKIs was not identified (**Figure 1**).

On-target resistance mechanisms: MET mutations and amplification

Focusing on MET-dependent resistance mechanisms, secondary *MET* kinase domain mutations in residues H1094, G1163, L1195, D1228, and Y1230 were acquired in 6 cases, and high focal amplification of the *MET* exon 14 mutant allele was identified in one case (summarized in **Table 1** and **supplementary Table 2**). Single *MET* resistance mutations were identified in four cases: D1228H in one patient (case #1), D1228N in two patients (cases #2 and #6), and Y1230C in one patient (case #3)

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1 **(Figure 2A)**. Multiple *MET* kinase domain mutations were detected in two cases. In an
2 updated analysis from case #4, which we previously described (9), after acquired
3 resistance to crizotinib, tissue showed only a Y1230H mutation, but plasma NGS
4 detected multiple MET kinase domain resistance mutations including G1163R, L1195V,
5 D1228N, D1228H, Y1230H, and Y1230S (**Figure 2B, supplementary Figure 2A)**. In
6 this case, by plasma NGS analysis, D1228 and Y1230 mutations were confirmed to be
7 in trans on different alleles, but the allelic distribution of the G1163R and L1195V
8 mutations relative to the other mutations could not be determined due to their distance
9 from the other genomic alterations (**Supplementary Figure 3**). In case #5, after
10 treatment with the type II MET TKI glesatinib, tissue NGS detected a MET H1094Y
11 mutation, however plasma NGS detected both the H1094Y and L1195V mutations
12 (**Figure 2B, supplementary Figure 2B**). The H1094Y substitution (also referred to as
13 H1112Y when referencing the *MET* variant 1 transcript, which is 18 amino acids longer
14 (32)) is an activating *MET* kinase domain mutation commonly found in papillary renal
15 cell carcinoma (33), but its impact on TKI resistance is unknown. By immunoblot
16 analysis, we found that *MET* H1094Y modestly reduces the ability of glesatinib to
17 dephosphorylate MET compared to wild type H1094 (**supplementary Figure 4**).
18 Interestingly, the H1094Y mutation appeared to be particularly sensitive to the type I
19 MET TKI savolitinib compared to wild type H1094 *MET* (**supplementary Figure 4**). By
20 structural modelling, this is likely due to the increased hydrophobic interaction between
21 Y1094 and the imidazo-pyridine head group of savolitinib. Molecular dynamics
22 simulation and binding free energy calculations indicate that H1094Y mutation
23 increases the binding energy to savolitinib by 3.7 kcal/mol. In contrast, H1094Y reduces

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the binding affinity of glesatinib by 2.7 kcal/mol. Due to the distance between nucleotides encoding for H1094Y and L1195V, it was not possible to determine whether these mutations were present *in cis* or *in trans* by phasing analysis on plasma NGS.

In addition to secondary MET kinase domain mutations, on-target mechanisms of resistance also involved high levels of MET amplification. Prior to treatment with MET TKIs, case #7 had an estimated 4 copies of *MET* at baseline, but upon acquired resistance to the type II MET TKI glesatinib, 17 copies of the *MET* exon 14 mutant allele were detected. After subsequent treatment with crizotinib after glesatinib, this case developed further amplification of the MET exon 14 mutant allele (up to 47 copies) (**Figure 2B, Figure 4B**).

Off-target resistance mechanisms

Acquired amplification of the ERBB family of receptor tyrosine kinase genes *EGFR* and *HER3,* and amplification of the mitogen-activated protein kinase (MAPK) pathway effector genes *KRAS* and *BRAF*, as well as acquired activating *KRAS* mutations were recurrently found at resistance to MET TKIs (**Figure 3, supplementary Table 3**). As a single event, *EGFR* amplification was detected in one case (#9), and *KRAS* amplification in a second case (#13) (**supplementary Figure 5**). Amplification of more than one gene was also commonly detected, including: *KRAS*/*EGFR* amplification in two patients (cases #10 and #12, **Figure 3A**), *EGFR*/*HER3* amplification in two patients (case # 6, **Figure 2B** and case #8 **Figure 3A**), and *KRAS*/*BRAF*/*EGFR*/*MET* amplification in one case (#11 **Figure 3A, supplementary Figure 5D**). We previously

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reported three of these cases (cases #10, #11, #13) in an earlier description of bypass mechanisms of resistance to MET TKIs in MET exon 14 mutant NSCLC (17).

KRAS mutations were also implicated in resistance to MET TKIs. A *KRAS* G12D mutation was acquired in one case driving resistance to crizotinib (#14, **Figure 3B**). In a second patient previously treated with crizotinib, chemotherapy, and nivolumab, for which no other resistance mechanisms were found in tissue NGS, a *KRAS* G60D mutation was found in plasma NGS prior to treatment with glesatinib. This patient received treatment with a type II MET TKI but did not experience a clinical benefit (#15, **Figure 3B** and **supplementary Figure 2C**). The *KRAS* G60D mutation was not detected by tissue NGS at the time of progression to glesatinib.

In 5 cases (25%), NGS of post-treatment samples did not detect genomic alterations with a clear role in resistance to MET TKIs **(supplementary Table 4, supplementary Figure 6)**.

Overcoming acquired resistance with sequential MET TKI treatment

We next examined the efficacy of sequential treatment with MET TKIs in six patients, according to the resistance mechanisms acquired with the previous line of MET inhibition. Four patients received a type I followed by a type II MET TKI: crizotinib followed by glesatinib in two patients, crizotinib followed by merestinib in one patient, and capmatinib followed by merestinib in one case. In addition, two patients received sequential treatment with glesatinib (type II) followed by crizotinib (type I) (**supplementary Table 1**).

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1 Overall, four of the six patients had detectable on-target resistance mechanisms
2 prior to switching treatment (Cases #3-5 and #7, **Figure 2B**). In a fifth case, with
3 resistance to capmatinib, *EGFR* and *HER3* amplification were detected on tissue
4 whereas the *MET* D1228N mutation was found in plasma only (Case #6). In a sixth
5 patient, a *KRAS* mutation was present at the time of treatment initiation with the second
6 MET TKI (Case #15, **Figure 3B**).

7 Sequential treatment with a structurally different MET TKI was effective in
8 overcoming single on-target resistance mechanisms in two patients (2/6, 33%). In one
9 patient with an acquired crizotinib-resistant *MET* Y1230C mutation detected in both
10 tissue and plasma, switching treatment to merestinib resulted in a confirmed partial
11 response (case #3, **Figure 4A**). In case #7, the patient's cancer developed focal
12 amplification of the *MET* exon 14 mutant allele (from 4 to 17 copies) at the time of
13 glesatinib resistance, and after switching treatment to crizotinib, the patient experienced
14 a confirmed partial response lasting for 10 months (**Figure 4B)**. However, at the time of
15 crizotinib resistance, higher levels of amplification of the *MET* exon 14 mutant allele (47
16 copies) were detected, suggesting that incremental levels of focal *MET* amplification
17 can confer resistance to both type I and II MET TKIs.

18 In four patients, sequential treatment with an alternative MET TKI did not result in
19 clinical benefit. Among these, in two patients, multiple *MET* mutations were found at the
20 time of progression on the first MET TKI treatment. One patient (case #4) with acquired
21 resistance to crizotinib not only had multiple secondary *MET* kinase domain mutations
22 that confer resistance to crizotinib (D1228N/H, Y1230S/H and G1163R), but also had a
23 *MET* L1195V mutation detected at a low allele fraction of 0.04%, and this mutation has

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1 been reported to confer resistance to both crizotinib and type II MET TKIs *in vitro*
2 (**Figure 2B**, **supplementary Figure 2A**)(15). Switching treatment to glesatinib was
3 ineffective, and higher levels of the MET L1195V mutation were detected by plasma
4 monitoring during treatment with glesatinib and at the time of progression (allelic
5 frequency increased from 0.04% to 0.8%) (case #4, **supplementary Figure 2A**). In the
6 second patient, sequential treatment with crizotinib after progression on glesatinib in the
7 context of both *MET* L1195V and H1094Y mutations was also ineffective (case #5,
8 **supplementary Figure 2B**), highlighting the challenges of targeting the L1195V
9 mutation due to its broad impact on sensitivity to both crizotinib and type II MET TKIs.
10 In the remaining two cases harboring off-target resistance mechanisms (cases
11 #6 and #14), sequential MET treatment strategies were ineffective. In one patient (case
12 #6, **Figure 2B**) in which *EGFR* and *HER3* amplification (detected by tissue NGS) and a
13 *MET* D1228N mutation (detected only by plasma NGS) were found at capmatinib
14 resistance, primary progression occurred in the lungs after switching treatment to
15 merestinib. At merestinib progression, the *MET* D1228N mutation was no longer
16 detected by plasma NGS, suggesting that merestinib was active against *MET* D1228N
17 mutant clones, but resistance was most likely driven by the co-occurring *EGFR* and/or
18 HER3 bypass signaling (case #6, **Figure 2B**). In another patient (case #14, **Figure 3B**),
19 who had a partial response to crizotinib as the first MET TKI lasting for 13 months, no
20 resistance mechanisms were detected on tissue or blood NGS. However, after
21 treatment with chemotherapy and immunotherapy, a *KRAS* G60D mutation was
22 detected in plasma NGS (AF: 1.08%) prior to starting treatment with glesatinib.
23 Switching to the type II MET TKI resulted in a short disease stability lasting for only 3

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months with persistence of the KRAS mutation by plasma monitoring at progression (case #15, **supplementary Figure 2C**).

Concordance between plasma and tissue NGS to asses resistance to MET TKIs

We compared the detection rate of genomic resistance mechanisms in both plasma and tissue NGS in eleven paired samples. Concordant results, defined as the detection of genomic alterations in plasma and tissue NGS, were observed in three cases (**supplementary Figure 2D**). Discordant results were observed in six cases (55%): in two patients, plasma NGS detected multiple *MET* kinase domain mutations whereas tissue NGS detected only single *MET* mutations; in one case a *KRAS* G60D mutation was detected in plasma but not in tissue, and in the remaining three cases, tissue NGS detected *MET* amplification or *EGFR* gene amplification which were not found in plasma NGS. Plasma NGS was not informative in 2 cases in which there was no evidence of tumor DNA shedding.

Clinical outcomes of patients treated with MET TKI and resistance mechanisms.

In *EGFR*-mutant lung cancer with acquired resistance to TKIs, clinical outcomes may differ depending on whether the cancers developed on-target versus off-target resistance (34,35); therefore, we also sought to explore clinical outcomes in our cohort based on MET TKI resistance mechanism. Among patients in which resistance to MET TKIs was assessed (N = 20), the median PFS to the first line of MET TKI was 6.7 months (95% CI: 4.7-29.4) and median time to treatment discontinuation (TTD) was 8.3 months (95% CI: 5.7-28.7). There were no significant differences in median PFS

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1 according to the type of resistance mechanisms: 7.4 months (95% CI: 5.2-15.9) in
2 patients with on-target versus 5.7 months (95%CI: 3.2-14.0) with off-target resistance
3 (p = 0.9) **(supplementary Figure 7A)**. TTD was also similar between groups, 8.3
4 months (95% CI: 5.9-21.7) versus 8.8 months (95% CI: 3.2-14.0), respectively (p = 0.4)
5 **(supplementary Figure 7B, supplementary Figure 8).**
6
7 **Discussion**

8 MET TKIs are expected to become a standard treatment option for patients with
9 *MET* exon 14 mutant lung cancer. The present study provides clinical and molecular
10 evidence suggesting that resistance driven by genomic alterations recurrently fall in two
11 broad categories: on-target resistance mediated by secondary *MET* kinase domain
12 mutations and/or amplification of the *MET* exon 14 mutant allele; and off-target
13 resistance resulting from activation of bypass signaling due to amplification of ERBB
14 family of receptor tyrosine kinase genes, BRAF amplification, *KRAS* amplification, and
15 *KRAS* mutations.

16 *MET* kinase domain mutations were frequently involved in resistance to type I
17 and type II MET TKIs as single or polyclonal events. Hotspot mutations in codons
18 D1228 and Y1230 were detected in about a third of patients experiencing disease
19 progression on a type I MET TKI. In concordance with preclinical studies showing
20 activity of type II inhibitors in this setting, we showed that resistance to crizotinib, driven
21 by the *MET* Y1230C mutation, was clinically targetable with merestinib (15,36).
22 However, in two cases, switching treatment to a type II MET TKI was not effective,
23 potentially hampered by the co-occurrence of alternative mechanisms of resistance like

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EGFR amplification or the selection of cancer subclones harboring the *MET* L1195V mutation, which confers resistance to crizotinib as well as to type II MET TKIs(15). The complex nature of on-target alterations reflects the diversity of subclonal selection under treatment pressure (37). Most of the on-target resistance mutations detected have been reported previously in studies that employed genomic data or *in vitro* modelling, in which clinical correlation was missing (15,38). Our study provides further information on the clinical context in which these mutations emerge and the efficacy of subsequent treatments with MET TKIs.

In one case, resistance to the type II MET TKI glesatinib appeared to be driven by high levels of focal amplification of the *MET* exon 14 mutant allele, and this patient had a subsequent response to the type I MET TKI crizotinib. One possible explanation for this response to crizotinib may be a difference in potency between TKIs. While a MET exon 14 mutant-expressing NIH/3T3 spheroid growth model showed greater potency with crizotinib compared to glesatinib (with a 50% inhibitory concentration [IC_{50}] of 28.9 nanomolar [nM] compared to IC_{50} 80.6 nM, respectively (9), by contrast, a Ba/F3 cell model suggests similar *in vitro* activity against MET exon 14 between crizotinib and glesatinib with IC_{50} values of 22 nM versus 21 nM, respectively (15). Alternatively, differences in pharmacokinetics or tolerability may have limited the maximal drug concentration of glesatinib compared to crizotinib; plasma levels of these drugs were not available for study in this case. Similar on-target amplification has been reported to drive resistance in *ALK* rearranged lung cancers, where *ALK* amplification can cause resistance to crizotinib but is not observed with more potent ALK TKIs (39). Hence, in light of the complex landscape of on-target molecular alterations, the optimal

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1 sequencing of MET-targeting strategy needs further prospective exploration through
2 clinical trials which also aim to identify the mechanism of resistance to the prior MET
3 inhibitor. In the setting of on-target resistance, additional MET-targeted strategies
4 employing MET antibodies or MET antibody-drug conjugates, should also be explored
5 prospectively through clinical studies (40–42).
6 Off-target genomic bypass mechanisms were detected in about half of the
7 patients in the present study. *MET* amplification is a common mechanism of resistance
8 to EGFR inhibitors in EGFR-mutant lung cancer, and combining MET and EGFR
9 inhibitors can be an effective strategy in overcoming this mechanism of resistance (43–
10 45). Given that focal *EGFR* amplification was frequently involved in resistance to type I
11 MET TKIs, exploring the role of dual MET and EGFR inhibition in this setting is
12 warranted (46). In concordance with previous reports, *KRAS* oncogenic mutations and
13 wild type *KRAS* and *BRAF* amplification constituted a common cause of resistance to
14 MET TKIs in our study (17,18). In the setting of oncogenic KRAS activation, preclinical
15 evidence suggests an additive effect of combining MET with MEK or EGFR/HER2
16 inhibition (18,47). Our results further support the need to explore optimal combination
17 strategies to delay or overcome resistance secondary to bypass activation of the MAPK
18 pathway (18).
19 As with other targeted therapy populations, we also observed discordance
20 between tissue and plasma genotyping (48,49). While plasma genotyping can
21 potentially reflect greater inter-tumoral heterogeneity of resistance than an analysis of a
22 single tumor biopsy site, some cancers do not shed sufficient quantities of tumor DNA
23 into the circulation to allow for accurate detection of resistance mechanisms using a

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1 blood sample alone (50). Furthermore, gene copy number gains and losses can be
2 more difficult to ascertain in plasma than in tissue (51), which could limit detection of
3 bypass mechanisms of resistance using plasma only. Additionally, plasma samples can
4 contain other sources of somatic mutations other than from the primary tumor of
5 interest, such as from clonal hematopoietic cell populations which can complicate the
6 elucidation of TKI-resistance mechanisms (52). With the various advantages and
7 disadvantages of plasma versus tissue analysis, we will need to continue exploring the
8 optimal sequencing platform that enables the most sensitive and specific identification
9 of resistance mechanisms safely and rapidly.

10 This study has several limitations. Firstly, genomic testing on tissue samples was
11 not performed with a single NGS assay in all patients. However, OncoPanel testing was
12 performed in most cases (87.5%). In addition, as only patients experiencing disease
13 progression were included in the study, the mechanisms of resistance we describe here
14 may encompass those occurring in a population of patients experiencing earlier disease
15 progression with MET TKI. Patients with long-term response to TKIs might not be
16 represented here and alternative mechanisms of resistance could develop in this
17 setting. Lastly, the sample size is relatively small and thus, the frequency of the reported
18 genomic alterations could vary in larger cohorts. Nevertheless, this is the largest study
19 to date exploring genomic mechanisms of resistance to MET TKI in the clinical setting.
20

21 **Conclusions**

22 Our study indicates that the mechanisms of resistance to MET TKIs are heterogeneous.
23 Systematic genomic profiling of tumor biopsies and plasma from patients progressing

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1 on treatment with MET TKIs can be informative in the treatment decision process and
2 can identify patients who might benefit from sequential MET inhibitor strategies and
3 contribute to the design of future clinical trials.
4

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1 **Table 1.** Summary of on-target *MET* mutations found upon resistance to each of the list
2 MET tyrosine kinase inhibitors (TKIs) used in this cohort.

MET TKI	Resistance mutations
Type Ia Crizotinib	G1163R D1228H/N Y1230C/H/S L1195V
Type Ib Capmatinib	D1228N
Type II Glesatinib	H1094Y L1195V

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1 **Figure Legends**

2 **Figure 1.** Distribution of on-target, off-target and unknown mechanisms of resistance to
3 MET tyrosine kinase inhibitors. # D1228N detected only in plasma and EGFR/HER3
4 amplification in tissue. * detected in plasma after previous treatment with crizotinib,
5 chemotherapy, and nivolumab.

6

7 **Figure 2.** Oncoprint of genomic alterations detected in tissue (T) and plasma (B) next-
8 generation sequencing in tumors harboring on-target resistance mechanisms to single
9 (**A**) or multiple lines (**B**) of MET tyrosine kinase inhibitors. Pre: pretreatment, R1:
10 resistance to first line of MET TKI, R2: resistance to second line of MET TKI, PR: partial
11 response, SD: stable disease, NE: not evaluable, TTD: time to treatment
12 discontinuation, NR: not reportable at the request of the sponsor. Low copy number
13 gain is defined as 3-5 copies and gene amplification is defined as ≥6 copies.

14

15 **Figure 3.** Oncoprint of genomic alterations detected in tissue (T) and plasma (B) next-
16 generation sequencing in tumors harboring off-target resistance mechanisms to single
17 (**A**) or multiple lines (**B**) of MET tyrosine kinase inhibitors. Pre: pretreatment, R1:
18 resistance to first line of MET TKI, R2: resistance to second line of MET TKI, PR: partial
19 response, SD: stable disease, NE: not evaluable, TTD: time to treatment
20 discontinuation, NR: not reportable at the request of the sponsor. Low copy number
21 gain is defined as 3-5 copies and gene amplification is defined as ≥6 copies.

22

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1 **Figure 4.** Clinical cases of effective sequential treatment with type I and type II MET
2 TKI. (**A**) A patient with extensive pleural disease (case #3) received treatment with
3 crizotinib as first line of MET TKI treatment. At the time of progression, both plasma and
4 tissue NGS detected a MET Y1230C mutations and switching treatment to merestinib
5 resulted in a rapid partial response. (**B**) Baseline levels of MET copy number are shown
6 (case #7), reflecting MET polysomy by NGS and fluorescence in situ hybridization
7 (FISH). After an initial partial response with glesatinib, MET amplification was detected
8 on tissue NGS (17 copies). Tumor responses were observed after switching treatment
9 to crizotinib lasting for 10 months. At the time of progression, a tissue biopsy showed
10 higher levels of MET amplification by NGS (47 copies) and FISH.
11
12
13

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Figure 1

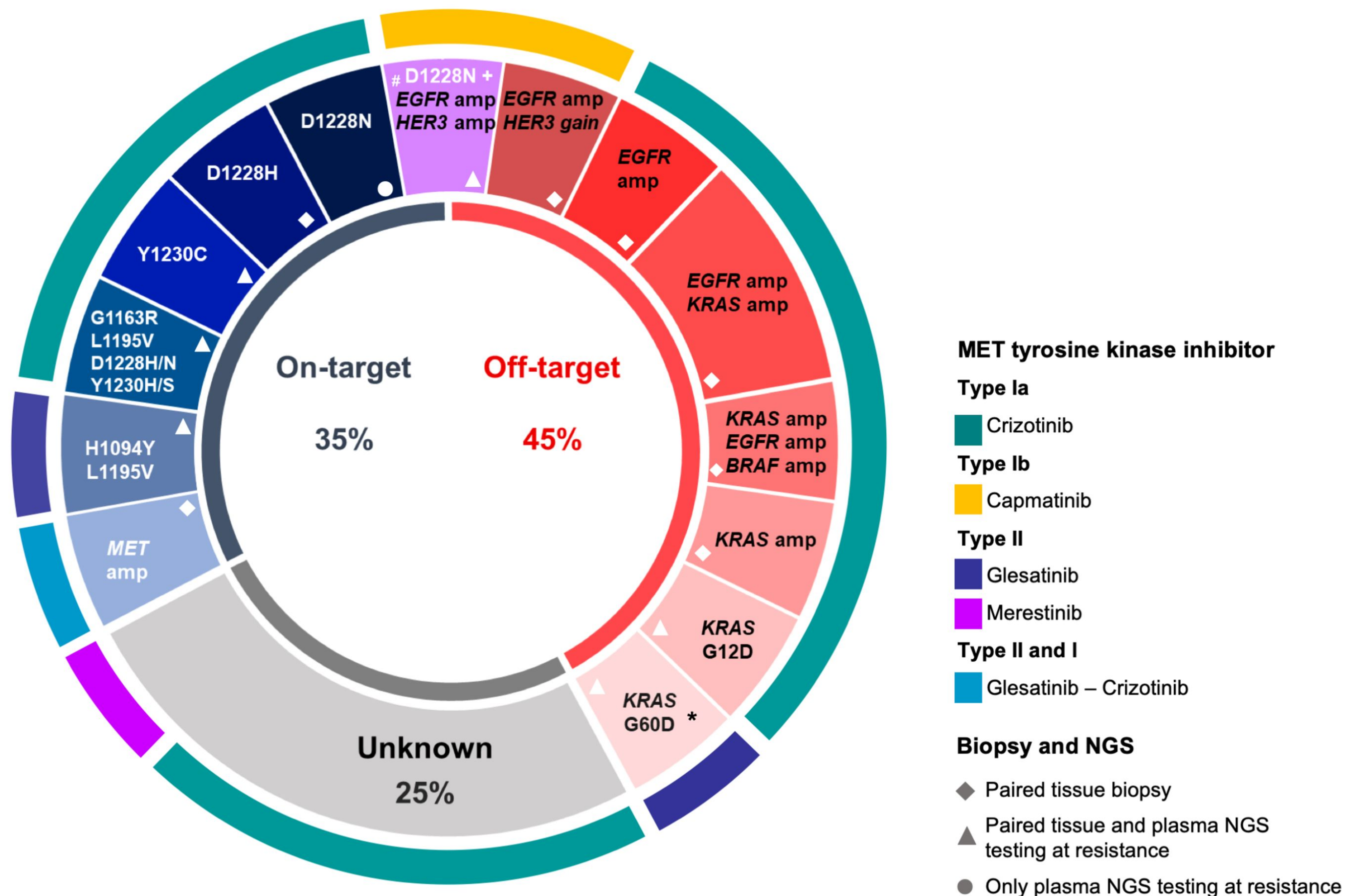

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Figure 2

A

Case #	Clinical	Drug	Response	TTD	Sample
1	Pre	-	-	-	T
	R1	Crizotinib	PR	10.8	T
2	Pre	-	-	-	T
	R1	Crizotinib	PR	5.9	B
3	Pre	-	-	-	T
	R1	Crizotinib	SD	15.9	T
					B

METex14, MET amp, MET S997*, H1094Y, G1163R, L1195F, L1195V, D1228H, D1228N, D1228V, Y1230C, Y1230H, Y1230S, EGFR, HER2, HER3, KRAS, BRAF, NF1, MEK1, PI3KCA, TP53, RB1, MDM2, CDK4, CDK6, CDKN2A/B, CCND1, CCNE1, CDKN1C

Splicing site mutation or frameshift mutation
Missense mutation
Low copy number gain
Amplification
Single copy deletion
Two-copy deletion
No alteration
Not assessed

B

Case #	Clinical	Drug	Response	TTD	Sample
4	Pre	-	-	-	T
	R1	Crizotinib	PR	7.4	T
					B
	Pre	Chemotherapy	NE	NE	B
	R2	Glesatinib	PD	1	B
5	Pre	-	-	-	T
	R1	Glesatinib	SD	21.2	T
					B
	R2	Crizotinib	PD	1.4	B
6	Pre	-	-	-	T
	R1	Capmatinib	NR	NR	T
					B
	R2	Meresetinib	PD	1.4	B
7	Pre	-	-	-	T
	R1	Glesatinib	PR	8.3	T
					B
	R2	Crizotinib	SD	10.3	T
					B

METex14, MET amp, MET S997*, H1094Y, G1163R, L1195F, L1195V, D1228H, D1228N, D1228V, Y1230C, Y1230H, Y1230S, EGFR, HER2, HER3, KRAS, BRAF, NF1, MEK1, PI3KCA, TP53, RB1, MDM2, CDK4, CDK6, CDKN2A/B, CCND1, CCNE1, CDKN1C

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Figure 3

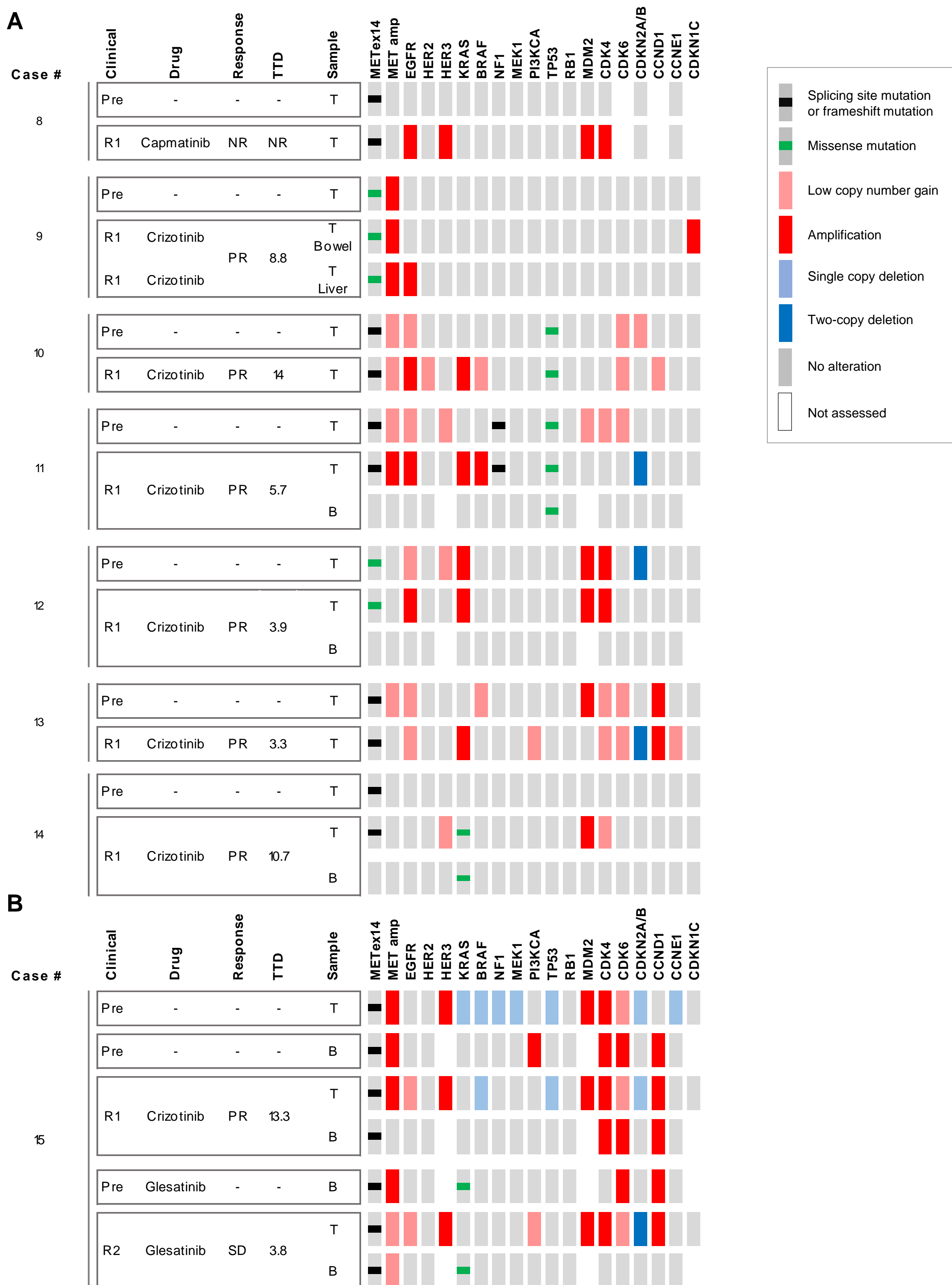

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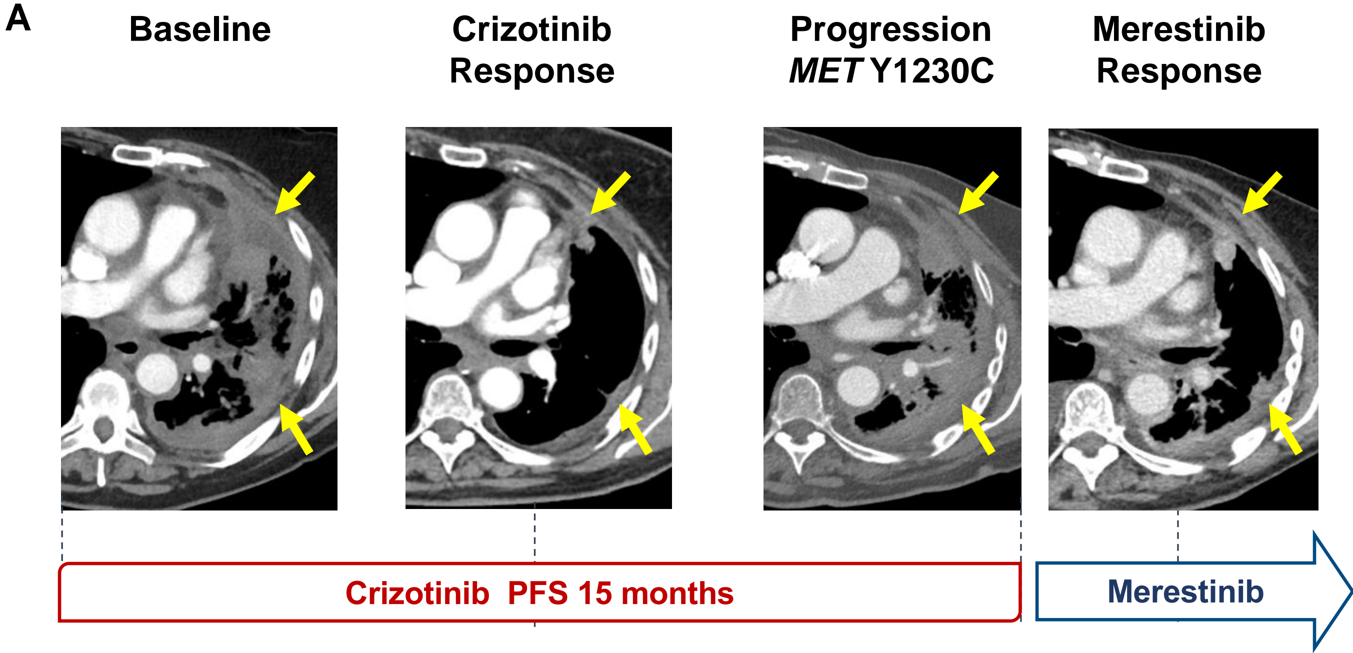
Figure 4



B

Baseline

Glesatinib Response

Glesatinib Resistance

Crizotinib Response

Crizotinib Progression

Glesatinib PFS 5.5 months

Crizotinib PFS 10.3 months

Adjusted copy number

MET 4 copies

MET 17 copies

MET 47 copies

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Gonzalo Recondo, Magda Bahcall, Liam F Spurr, et al.

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