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Original Study

Actionability of on-target *ALK* Resistance Mutations in Patients With Non-Small Cell Lung Cancer: Local Experience and Review of the Literature

Bart Koopman,[1] Harry J.M. Groen,[2] Ed Schuuring,[1] T. Jeroen N. Hiltermann,[2] Wim Timens,[1] Wilfred F.A. den Dunnen,[1] Anke van den Berg,[1] Arja ter Elst,[1] Michel van Kruchten,[3] Joost L. Kluiver,[1] Birgitta I. Hiddinga,[2] Lucie B.M. Hijmering-Kappelle,[2] Matthew R. Groves,[4] Juliana F. Vilacha,[4] Léon C. van Kempen,[1,#] Anthonie J. van der Wekken[2,#]

Abstract

***ALK*-fusion-positive NSCLC patients treated with ALK inhibitors frequently develop on-target resistance mutations. We provide clinical evidence for targeting these mutations with currently available inhibitors using a pooled population of 387 patients. The majority achieved clinical benefit, but the likelihood of clinical benefit differed for each mutation-inhibitor combination. Our comprehensive overview can facilitate guidance for treating similar patients in clinical practice.**

Introduction: Non-small cell lung cancer (NSCLC) patients with Anaplastic Lymphoma Kinase (*ALK*) gene fusions respond well to ALK inhibitors but commonly develop on-target resistance mutations. The objective of this study is to collect clinical evidence for subsequent treatment with ALK inhibitors. **Patients and Methods:** Local experience with on-target *ALK* resistance mutations and review of the literature identified 387 patients with ALK inhibitor resistance mutations. Clinical benefit of mutation-inhibitor combinations was assessed based on reported response, progression-free survival and duration of treatment. Furthermore, this clinical evidence was compared to previously reported *in vitro* sensitivity of mutations to the inhibitors. **Results:** Of the pooled population of 387 patients in this analysis, 239 (62%) received at least 1 additional line of ALK inhibition after developing on-target resistance to ALK inhibitor therapy. Clinical benefit was reported for 177 (68%) patients, but differed for each mutation-inhibitor combination. Agreement between *in vitro* predicted sensitivity of 6 published models and observed clinical benefit ranged from 69% to 89%. The observed clinical evidence for highest probability of response in the context of specific on-target ALK inhibitor resistance mutations is presented. **Conclusion:** Molecular diagnostics performed on tissue samples that are refractive to ALK inhibitor therapy can reveal new options for targeted therapy for NSCLC patients. Our comprehensive overview of clinical evidence of drug actionability of *ALK* on-target resistance mutations may serve as a practical guide to select the most optimal drug for individual patients.

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Keywords: ALK, Non-small cell lung cancer, Mutation, Resistance, Sensitivity

Introduction

Oncogenic fusions involving the Anaplastic Lymphoma Kinase gene (*ALK*) are detected in approximately four percent of patients with advanced non-small cell lung cancer (NSCLC).[1,2] In the majority of patients, an inversion of a small part of chromosome 2p causes a fusion of the amino-terminal coiled-coil domain of

[1]Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[2]Department of Pulmonary Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[3]Department of Medical Oncology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[4]Structural Biology in Drug Design, University of Groningen, Groningen Research Institute of Pharmacy, Groningen, The Netherlands

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Address for correspondence: Léon C. van Kempen, PhD Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, PO Box 30.001, 9700RB Groningen, The Netherlands.

E-mail contact: l.van.kempen@umcg.nl

[#] Both share senior authorship

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the echinoderm microtubule-associated protein-like 4 gene (*EML4*) with the tyrosine kinase domain of *ALK* (exons 20 through 29) (*EML4-ALK*).[1,3] Various other fusion partners have been reported.[4] Soon after the discovery of the *EML4-ALK* oncogene,[1] lung cancers harboring *ALK* fusions were shown to be highly sensitive to the ALK inhibitor crizotinib,[5] leading to its FDA approval in 2011.[6] Various other ALK inhibitors targeting the ATP-binding pocket of ALK have since been approved for the treatment of patients with *ALK* fusion-positive NSCLC, including second-generation inhibitors ceritinib,[7] alectinib, [8] and brigatinib,[9] and the third-generation inhibitor lorlatinib.[10]

Despite high response rates, ranging from 54% to 94%,[6-9] patients treated with ALK inhibitors ultimately experience disease progression.[11,12] Mutations in the *ALK* kinase domain, detected in approximately 19%-54% of ALK inhibitor resistant samples,[13-19] account for so-called on-target resistance. *In vitro* studies testing the efficiency of multiple on-target mutations to crizotinib, ceritinib, alectinib and brigatinib, indicated a distinct inhibitor specific efficacy spectrum.[13]

Due to the diversity in *ALK* mutations in second and subsequent treatment lines and the different types of available ALK inhibitors as well as the sequence in which patients receive these, it remains difficult to select the most optimal treatment for individual patients. Such patients are therefore commonly reviewed by Molecular Tumor Boards (MTBs),[20] whose advice largely depends on limited reports describing clinical drug effectiveness of specific *ALK* mutational profiles to available ALK inhibitors. Due to lack of clinical information, decision-making is often based on *in vitro* reports of the half maximal inhibitory concentration (IC_{50}) for a drug in relation to a specific resistance-mediating mutation.[21,22] Although various reports have described recommendations on drug actionability,[13,23-25] the methods and read-out parameters used to determine *in vitro* sensitivity as well as cut-off values to predict actionability differ widely. Commonly used read-out parameters include determination of ALK phosphorylation status,[13] tritium incorporation proliferation,[23] and cell viability assays (**Table ST1, Table ST2**),[24,25] which makes a comparison between these models difficult. Furthermore, validation of their predictive values in a clinical setting have not been performed.

In this study, we evaluated the clinical tumor response of ALK inhibitors directed towards different on-target *ALK* mutations of 14 *ALK* fusion-positive NSCLC patients treated at the University Medical Center Groningen (UMCG) and 373 cases published by others, as well as the predictive value of the currently reported preclinical data.

Materials and Methods

Retrospective Cohort of ALK Inhibitor Resistant Non-Small Cell Lung Cancer Patients

Between January 1, 2014 and June 19, 2020, patients treated with *ALK* fusion-positive NSCLC who relapsed on an ALK inhibitor and harbored one or more therapy-induced *ALK* mutations were identified using the pathology and MTB databases of the UMCG.[20] Some of these patients have been previously reported in a study detailing the effectiveness of MTB recommendations for NSCLC patients.[20] Molecular profiling via targeted NGS covering exons 22 (amino acid positions (AA) 1151-1172), 23 (AA 1173-1215) and exon 25 (AA 1260-1279), covering all frequently described hotspots for on-target resistance,[13] were performed in the ISO-NEN-15189:2012-accredited UMCG molecular pathology laboratory.[26]

Patient history and follow-up data were retrieved from the hospital's electronic health records. Parameters that were extracted included age, sex, tumor stage, tumor histology, molecular profile (including type of *ALK* fusion and *ALK* mutations), treatments received and, for each line of ALK inhibitor treatment, radiological best overall response (BOR) according to RECIST criteria 1.1,[27] progression-free survival (PFS), duration of response (DoR), duration of treatment (DoT), overall survival (OS), and molecular characteristics of each subsequent biopsy, if available. Baseline characteristics were determined at initiation of first-line ALK inhibition. Some of the patients have been reported in previous publications.[20,28]

All included patients provided informed consent. Clinical data processing was performed in accordance with the General Data Protection Regulation (EU) 2016/679. The biobank initiative was approved by the medical ethics committee of the UMCG (no. 2010/109) and made available for this study.[29]

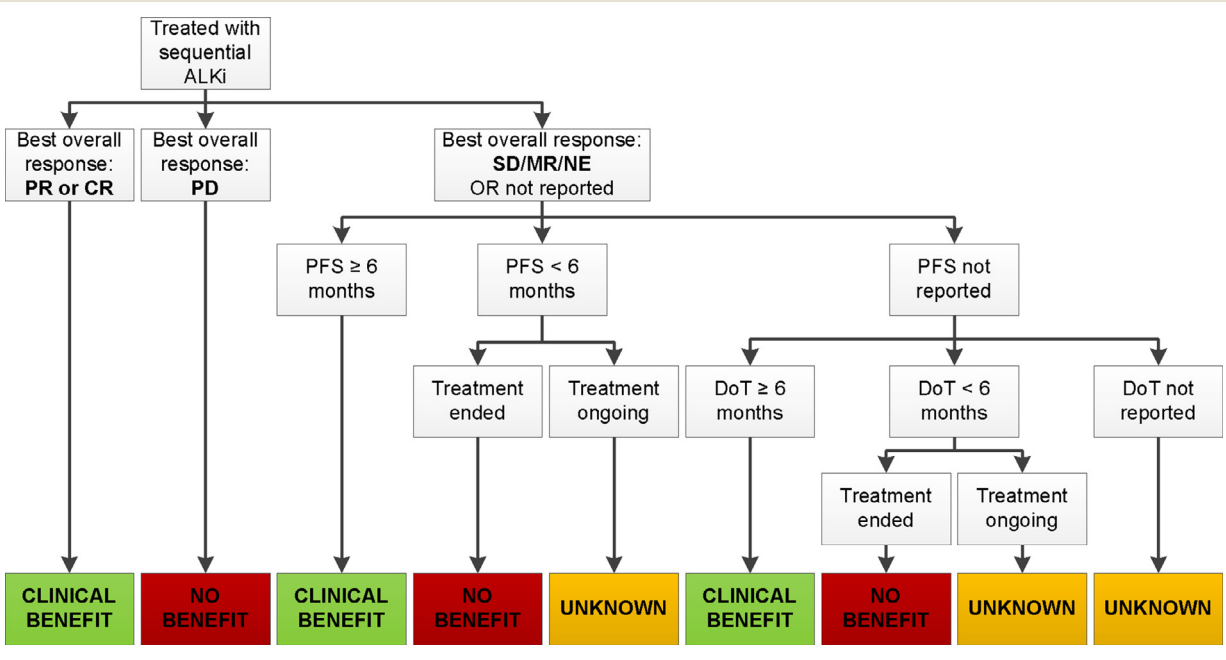
Literature Review: Actionability of ALK *Mutational Profiles in Non-Small Cell Lung Cancer*

A literature review was performed to identify clinical reports of individual *ALK* fusion-positive NSCLC patients previously treated with ALK inhibitors and harboring on-target mutations in *ALK* (details in *Supplementary Methods).* In total, 1469 articles (up to June 2020) were screened on title and abstract. Sixty-nine articles (36 cohort studies and 33 case reports) were included (*Table ST3*). For each patient, fusion variants, detected *ALK* mutations, variant allele frequencies (VAF), sample origin, and previously received ALK inhibitors were extracted and tabulated. If a patient was subsequently treated with an ALK inhibitor monotherapy, clinical response information was collected. Patients who were treated with combination therapy (such as chemotherapy and an ALK inhibitor) were excluded from the clinical response evaluation.

Assessment of Clinical Benefit Based On Available Treatment Results

The literature-derived *ALK* mutations and corresponding response data were pooled with those from our own academic center. Patients were classified as clinically benefiting from an ALK inhibitor in case of a documented complete or partial response (CR or PR) and not benefiting in case of progressive disease (PD). In case of stable disease (SD), mixed response (MR), not evaluable (NE) or unavailable BOR, patients were classified as benefiting if they demonstrated a PFS of ≥6 months. Patients were classified as not benefiting if PFS was less than 6 months and treatment had ended or "unknown" if treatment was reported to be ongoing. If PFS was not reported, DoT was used as a surrogate marker for PFS: patients were classified as benefiting if DoT of ≥6 months, and not benefiting if DoT of <6 months. If DoT was also not reported, patients' tumor response was classified as "unknown" (Figure 1). If a patient repre-

Figure 1 Assessment of clinical benefit based on available treatment results.
Abbreviations: ALKi = ALK inhibitor; CR = complete response; DoT = duration of treatment; MR = mixed response; NE = not evaluable; PD = progressive disease; PFS = progression-free survival; PR = partial response; SD = stable disease.



sented multiple longitudinal samples and was treated with a different ALK inhibitor for each new mutation, clinical benefit for each different mutation-inhibitor combination was assessed separately. The likelihood of clinical benefit was assessed for each mutation-inhibitor combination, taking into account the number of samples harboring a mutation after treatment with a specific inhibitor, as well as the amount of patients responding to that inhibitor while harboring the specific mutation.

Comparison of in vitro *Models of Sensitivity to Clinical Benefit*

Studies reporting on *in vitro* sensitivity of ALK inhibitors in relation to specific resistance-mediating mutations were identified using the same search strategy described in *Supplementary Methods*. The reported sensitivity for each mutation-inhibitor combination was extracted and classified as "sensitive" or "resistant" according to thresholds defined in each individual study. The *in vitro* 'prediction' of sensitivity was compared to the likelihood of clinical benefit derived from clinical responses in our study. For each individual model, the agreement was defined as the percentage of mutation-inhibitor combinations reported by that study that was in agreement with the likelihood of clinical benefit as defined in our study.

Statistics

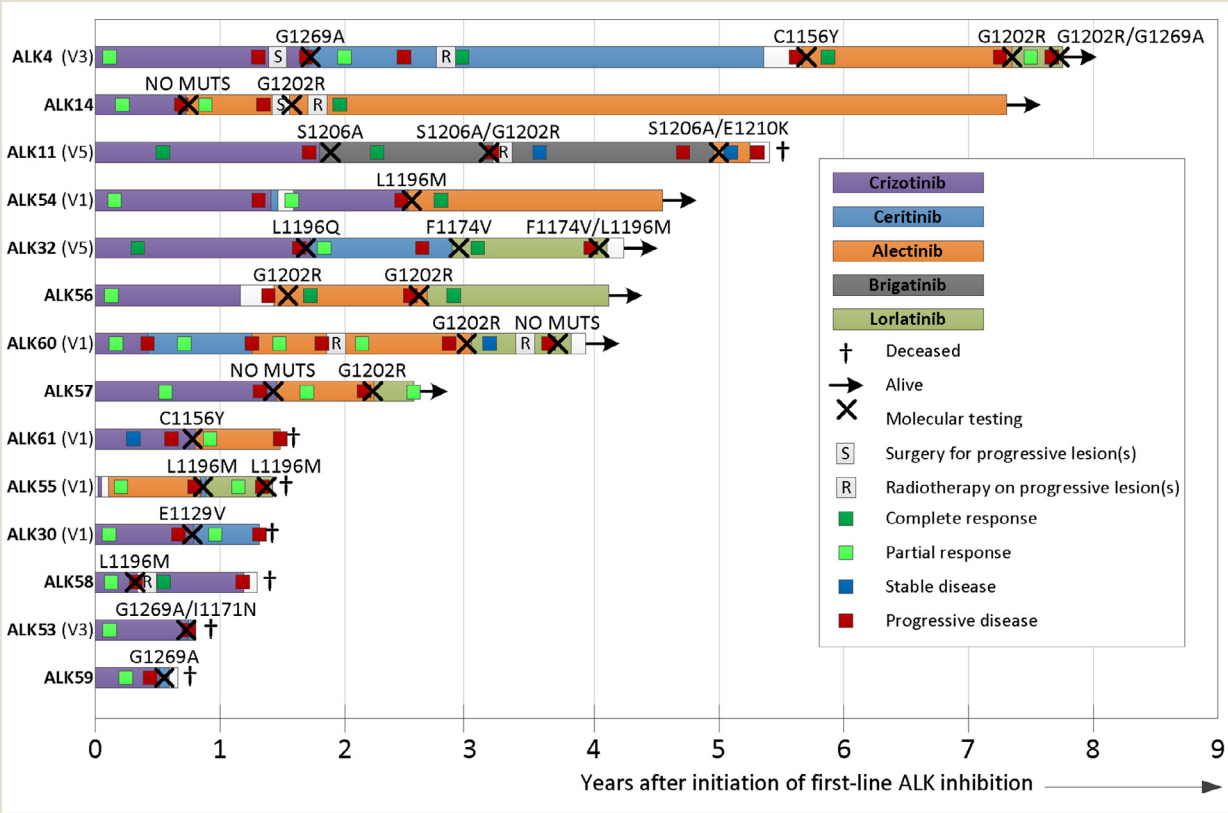
Descriptive statistics were used. Fisher's exact test was used to compare categorical variables. If correction for potential confounders was required, logistic regression was used. Results were considered significant at P values below .05. Statistical analyses were performed with SPSS version 23 (SPSS Inc.; Chicago, IL). Due to major differences in *in vitro* methods to assess drug sensitivity as well as differences in which variants were assessed, concordance statistics to compare the different *in vitro* reports to clinical results were deemed unreliable and therefore not performed.

Results

Characteristics of Patients With ALK Inhibitor Resistance Treated at the UMCG

Twenty-nine *ALK* fusion-positive NSCLC patients with resistance to first-line ALK inhibitor were tested 1 or more times for presence of on-target resistance mechanisms. Fourteen patients (48%) harbored *ALK* mutations in one or subsequent tissue samples, representing a total of 23 samples with *ALK* mutations. Clinical characteristics are presented in *Table ST4*. Clinical responses are summarized in Figure 2 and *Table ST5*. Twelve ALK inhibitor-resistant patients were treated with 1 or more additional lines of ALK inhibitors. Response was assessed for each line of treatment, which included ALK inhibitors in second line ($n = 12$), third line ($n = 4$) and fourth line ($n = 1$). An objective response was achieved in 71% (12/17). Median PFS was 9.2 months (95% confidence interval, 3.8-14.6). Objective responses were observed in the majority of patients for alectinib (60%; 3/5), brigatinib (CR lasting >16 months in 1patient), ceritinib (60%; 3/5) and lorlatinib (83%; 5/6)

Figure 2 Clinical course of 14 *ALK* fusion-positive NSCLC patients with *ALK* mutation(s) in UMCG.
Swimmer's plot illustrating the clinical course (in years on the x axis) after initiation of first-line ALK inhibition of *ALK* fusion-positive NSCLC patients with *ALK* mutation(s). Patients are indicated on the y axis (if known, *EML4-ALK* variant between brackets), ordered by overall survival. ALK inhibitors are color-coded and plotted in time; no active ALK inhibition is indicated by white bars. Treatment of progressive lesions with radiotherapy (R) or surgery (S) is indicated. Best overall radiological responses and molecular testing results are displayed at their respective points in time as colored blocks and black cross, respectively. Vital status is displayed at the end of each plot. Additional information can be found in Table ST4 and Table ST5. Some of the mutations in patients ALK11, ALK30, ALK53, ALK54, ALK55, and ALK56 were previously reported by Koopman et al. (2020), corresponding to patient numbers 13, 16, 5, 12, 18 and 30.[20] Furthermore, the first mutation in patient ALK4 was previously reported by Wei et al.[28]
Abbreviations: NO MUTS = no mutations detected; V1 = *EML4-ALK* variant 1 (*EML4* exon 13 fused to *ALK* exon 20); V3 = *EML4-ALK* variant 3 (*EML4* exon 6 fused to *ALK* exon 20); V5 = *EML4-ALK* variant 5 (*EML4* exon 2 fused to *ALK* exon 20).



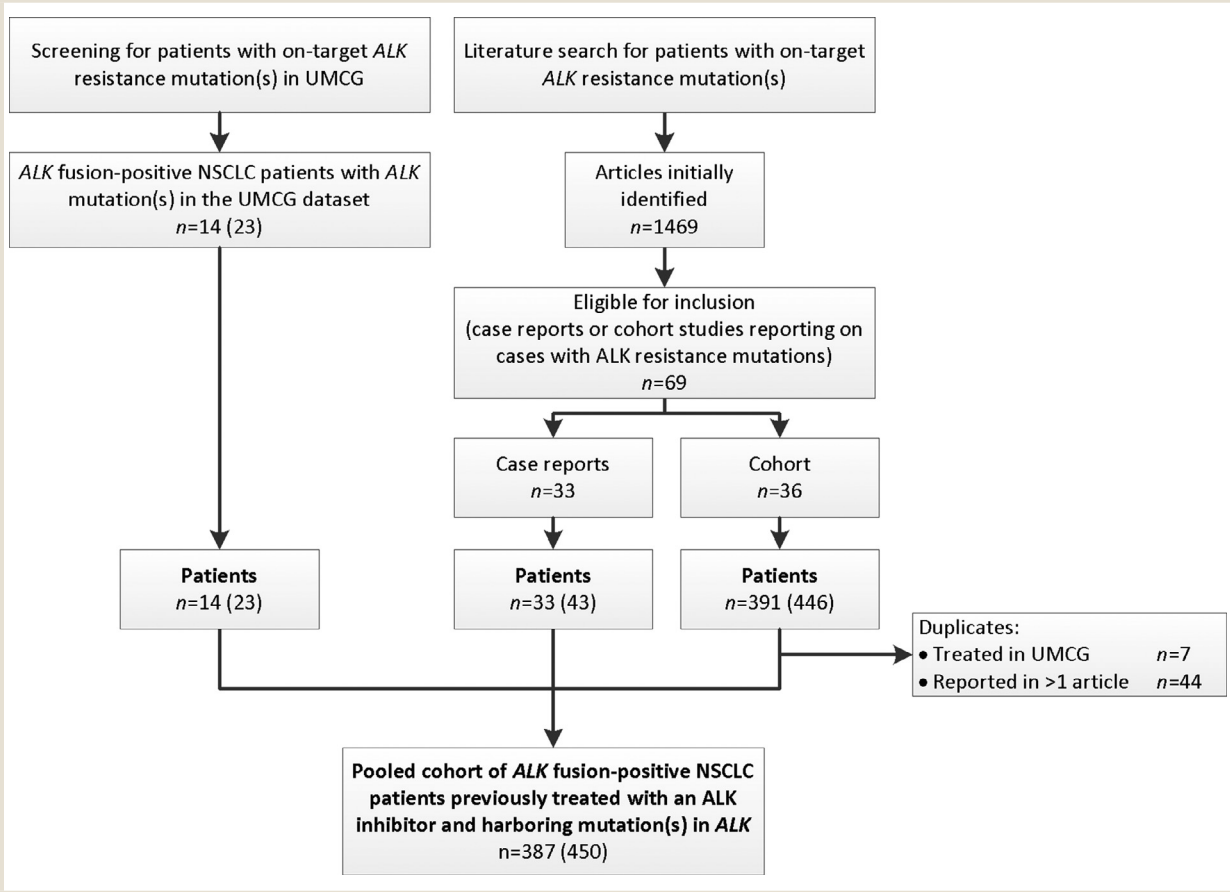
Literature Review to Identify Clinical Evidence on Drug Efficacy in ALK *Resistance Mutations*

A literature review identified 423 eligible *ALK* fusion-positive NSCLC patients: 390 patients with acquired *ALK* mutations reported in cohort studies and 33 patients from case reports. Duplicates were excluded: this included published patients that had been treated in the UMCG in prior reports ($n = 7$) and patients reported more than once by other groups ($n = 44$), leaving 373 unique patients. These patients were pooled with the 14 from the UMCG, resulting in 387 *ALK* fusion-positive NSCLC patients with therapy-induced *ALK* mutations in 450 biopsies (Figure 3 and *Table ST6*). Induced *ALK* on-target resistance patterns were obtained after monotherapy with any ALK inhibitor (Figure 4A).

Impact of Previous ALK Inhibitors and Fusion Partners on the Distribution of Acquired ALK *Mutations*

Of the 450 ALK inhibitor-pretreated samples, 345 (77%) harbored a single on-target mutation and 105 (23%) harbored multiple on-target mutations. A marked difference was observed in the frequency of resistance mutations when stratifying according to ALK inhibitor administered prior to the biopsy. Resistance to first-line crizotinib ($n = 220$) was associated with a wide range of individual mutations, with L1196M ($n = 55$ [25%]) and G1269A ($n = 34$ [15%]) occurring most frequently (Figure 4B). These on-target mutations were observed more often with crizotinib than with other inhibitors ($P < .001$ for both). Substitutions of I1171 ($n = 27$ [33%]) and G1202R ($n = 26$ [32%]) were the most common resistance-inducing mutations following treatment with

Figure 3 **Patient selection**
Flow chart depicting selection of patients. Depicted are patients treated at the UMCG (left column) and patients identified in literature (right column), with number of *ALK*-mutant samples presented between brackets. Fourteen patients (representing 23 samples) were treated in the UMCG. An additional 423 patients (390 from 36 cohort studies and 33 from single-case reports) were identified in literature. Confirmed duplicate reports on the same patients (*n*=50) were excluded, leaving 387 patients for inclusion, representing a total of 450 *ALK*-mutant samples.
Abbreviations: NSCLC = non-small cell lung cancer = UMCG, University Medical Center Groningen.



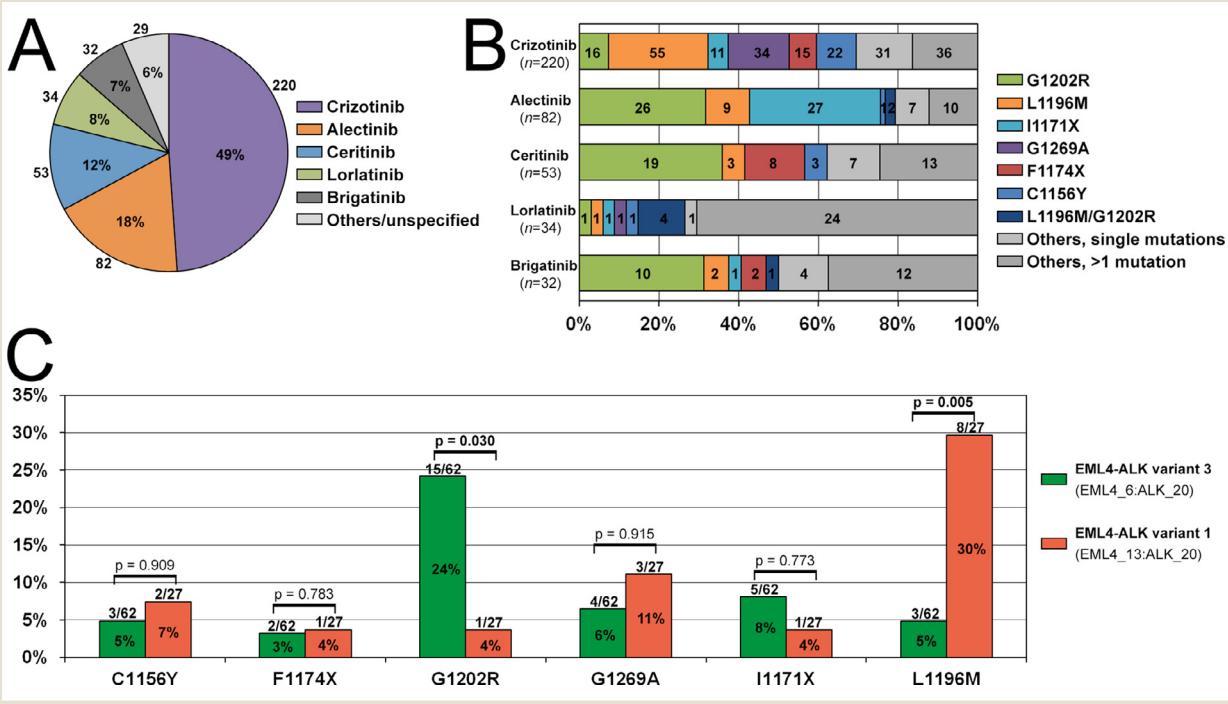
alectinib ($n = 82$). I1171X was more common with alectinib than with other inhibitors ($P < .001$). The on-target resistance mutations for ceritinib ($n = 53$) and brigatinib ($n = 32$) were comparable ($P = .54$), with G1202R as the most prevalent mutation ($n = 19$ [36%] and $n = 10$ [31%], respectively). The third-generation ALK inhibitor lorlatinib ($n = 34$) had a very different spectrum of resistance-induced mutations, with the vast majority of samples ($n = 24$ [82%]) harboring more than one *ALK* mutation. The most common on-target resistance mechanism for lorlatinib was the L1196M/G1202R compound mutation ($n = 4$ [12%]).

Differences in the type of *ALK* mutations reported in the 450 samples were not only drug-related but also fusion variant related. The *ALK* fusion partner was specified for 28% (107/387) of patients, with the vast majority harboring an *EML4-ALK* fusion ($n = 103$). The exact breakpoint was reported for 89 patients. The most common *ALK* fusion variants were *EML4-ALK* variant 3 ($n = 62$) and *EML4-ALK* variant 1 ($n = 27$). When corrected for previously administered ALK inhibitor, the G1202R mutation was more common in variant 3 than in variant 1 (24% vs. 4%; $P = .030$) (Figure 4C, *Table ST5*). On the contrary, L1196M was more prevalent in variant 1 than in variant 3 (30% vs. 5%; $P = .005$). The other commonly reported mutations, C1156Y, G1269A, F1174C/L/V and I1171N/S/T, were similarly distributed between both variants.

Clinical Benefit for ALK *Resistance Mutations From Sequential ALK Inhibitors*

Sequential treatment with a different ALK inhibitor to overcome acquired resistance due to *ALK* mutation(s) was reported for 239 patients (overview in *Table ST7*). If a patient represented multiple longitudinal samples and was treated with a different ALK inhibitor for each new mutation, clinical benefit for each different mutation-inhibitor combination was assessed separately. This resulted in 262 evaluations of tumor response to sequential treatment. Clinical benefit was confirmed in 177 (68%) response evaluations. Those with a single mutation were more likely to respond to treatment (70%; 150/213) than those with ≥2 mutations (55%;

Figure 4 Characteristics of the pooled population of 450 ALK inhibitor resistant, *ALK* fusion-positive NSCLC patients with acquired *ALK* mutations.
(A) Distribution of ALK inhibitors on which patients relapsed. (B) Distribution of *ALK* mutational resistance profiles according to the ALK inhibitor on which they relapse. (C) Differences in the prevalence of six most common single *ALK* mutations, organized by the two most common variants, *EML4-ALK* variant 3 (*EML4* exon 6 fused to *ALK* exon 20, *n*=62) and *EML4-ALK* variant 1 (*EML4* exon 13 fused to *ALK* exon 20, *n*=27). These results were corrected for previously received ALK inhibitor using logistic regression. Of note, the *ALK* fusion variant was not specified in the vast majority of patients in this study.
Abbreviations: NSCLC = non-small cell lung cancer.



27/49) (P = .044). The clinical benefit rate was not influenced by the reported number of previously received ALK inhibitors: 65% (109/168) in case of a single previous ALK inhibitor vs. 70% (50/71) in case of ≥2 previous ALK inhibitors (P = .455).

We summarized the likelihood of clinical benefit based on this pool of 450 *ALK*-mutant samples for the most frequently reported *ALK* resistance profiles in Figure 5, with more in-depth information in *Table ST8*. The clinical benefit was assessed for each mutation-inhibitor combination, based on reported clinical evidence only. Furthermore, the number of samples harboring a mutation after treatment with a specific inhibitor, as well as the amount of patients responding to that inhibitor while harboring the specific mutation were taken into account to determine the probability of clinical benefit (thresholds defined in Figure 5). This analysis revealed that the likelihood of clinical benefit differs for each mutation-inhibitor combination. Mutations affecting the same amino acid can either confer similar likelihood of benefit for the different ALK inhibitors (for example, substitutions of F1174), but also may represent differences in benefit (for example, substitutions of I1171). The likelihood of clinical benefit could not be corrected for fusion variant due to small sample sizes and because the fusion variant was not reported in the majority of cases.

Comparison of ALK Inhibitor Sensitivity as Predicted by in vitro *Assays to Observed Clinical Responses*

Six preclinical models of *in vitro* sensitivity of ALK inhibitors in relation to specific resistance-mediating mutations have been reported in four studies, which we dubbed Fontana-V3, Gainor-V1, Yoda-V1, Yoda-V3, Horn-V1 and Horn-V3 based on the first author and *EML4-ALK* fusion variant tested.[13,23-25] Although these reports all demonstrated the prediction of sensitivity or resistance for inhibitor-mutation combinations, there are major differences in methods used and types of *ALK* fusions, mutations and inhibitors tested (*Table ST1*), making it hard to directly compare the various models. In addition, 152 of the 450 *ALK*-mutant samples (34%) had mutations that were not tested by any of the six models on their potential sensitivity to ALK inhibitors.

Both clinical and *in vitro* findings can be useful for directing treatment decision-making.[20] Therefore, the observed clinical responses to ALK inhibitors for the different mutational profiles (Figure 5 and *Table ST8*) were compared to the predicted efficacies resulting from previously reported preclinical models (*Table ST2*). In total, there were 115 mutation-inhibitor combinations with *in vitro* evidence in at least 1 of the 6 models. Figure 6 summarizes clinical as well as *in vitro* evidence, to allow a head-to-head compar-

Figure 5 **Summary of clinical benefit deduced from tumor response and relapse with second and further lines of ALK inhibitor treatments on specific on-target resistance-inducing *ALK* mutations**
A cross-tabulation summarizing the number of patients with tumor response and those who relapsed on a specific ALK inhibitor for the most common ALK inhibitor-resistant *ALK* mutational profiles ($n \geq 6$). This cross-tabulation is based on reported clinical evidence only. Definition of expected clinical benefit is based on 2separate criteria: the percentage of patients treated with a specific inhibitor in first line who developed the specified mutation ("Relapsing") and the percentage of patients with a specific mutation who achieved clinical benefit when treated with the specified inhibitor ("Sensitivity"). These percentages are combined to classify a mutation-inhibitor combination as "Likely beneficial", "Possibly beneficial", "No benefit expected", "Conflicting evidence", or "Insufficient evidence", according to the scheme displayed below the cross-tabulation. Clinical benefit is defined in Methods. A detailed version of this table, which includes every reported combination of inhibitor - *ALK* mutations, can be found in *Table ST7*.

	Alectinib	Brigatinib	Ceritinib	Crizotinib	Lorlatinib
C1156Y	**Likely beneficial** Relapsing: **1/82 (1%)** Sensitivity: **5/5 (100%)**	Possibly beneficial Relapsing: **0/32 (0%)** Sensitivity: **1/1 (100%)**	Possibly beneficial Relapsing: **3/53 (6%)** Sensitivity: **2/3 (67%)**	No benefit expected Relapsing: **22/220 (10%)** No patients treated	Insufficient evidence Relapsing: 1/34 (3%) Sensitivity: 1/2 (50%)
I1171N	No benefit expected Relapsing: **17/82 (21%)** No cases treated	No benefit expected Relapsing: **1/32 (3%)** Sensitivity: **0/3 (0%)**	**Likely beneficial** Relapsing: **0/53 (0%)** Sensitivity: **6/6 (100%)**	Insufficient evidence Relapsing: 0/220 (0%) No patients treated	Possibly beneficial Relapsing: **1/34 (3%)** Sensitivity: **1/1 (100%)**
I1171S	No benefit expected Relapsing: **6/82 (7%)** No cases treated	Possibly beneficial Relapsing: **0/32 (0%)** Sensitivity: **1/1 (100%)**	Insufficient evidence Relapsing: 0/53 (0%) No patients treated	Insufficient evidence Relapsing: 1/220 (0.5%) No patients treated	Insufficient evidence Relapsing: 0/34 (0%) No patients treated
I1171T	Possibly beneficial Relapsing: **4/82 (5%)** Sensitivity: **2/2 (100%)**	No benefit expected Relapsing: **0/32 (0%)** Sensitivity: **0/1 (0%)**	**Likely beneficial** Relapsing: **0/53 (0%)** Sensitivity: **3/3 (100%)**	Insufficient evidence Relapsing: 10/220 (4%) No patients treated	Insufficient evidence Relapsing: 0/34 (0%) No patients treated
F1174C	Possibly beneficial Relapsing: **0/82 (0%)** Sensitivity: **1/1 (100%)**	Insufficient evidence Relapsing: 0/32 (0%) No patients treated	Possibly beneficial Relapsing: **3/53 (6%)** Sensitivity: **1/1 (100%)**	No benefit expected Relapsing: **2/220 (0.9%)** Sensitivity: **0/1 (0%)**	Possibly beneficial Relapsing: **0/34 (0%)** Sensitivity: **1/1 (100%)**
F1174L	Possibly beneficial Relapsing: **0/82 (0%)** Sensitivity: **1/1 (100%)**	Possibly beneficial Relapsing: **1/32 (3%)** Sensitivity: **1/1 (100%)**	No benefit expected Relapsing: **3/53 (6%)** Sensitivity: **0/1 (0%)**	Insufficient evidence Relapsing: 9/220 (4%) No patients treated	Insufficient evidence Relapsing: 0/34 (0%) No patients treated
F1174V	Possibly beneficial Relapsing: **0/82 (0%)** Sensitivity: **1/1 (100%)**	Insufficient evidence Relapsing: 1/32 (3%) No patients treated	Insufficient evidence Relapsing: 2/53 (4%) No patients treated	Insufficient evidence Relapsing: 4/220 (2%) No patients treated	Possibly beneficial Relapsing: **0/34 (0%)** Sensitivity: **2/2 (100%)**
L1196M	Conflicting evidence Relapsing: **9/82 (11%)** Sensitivity: **9/11 (82%)**	No benefit expected Relapsing: **2/32 (6%)** Sensitivity: **1/2 (50%)**	Possibly beneficial Relapsing: **3/53 (6%)** Sensitivity: **10/10 (100%)**	No benefit expected Relapsing: **55/220 (25%)** No cases treated	**Likely beneficial** Relapsing: **1/34 (3%)** Sensitivity: **7/10 (70%)**
G1202R	Conflicting evidence Relapsing: **26/82 (32%)** Sensitivity: **4/6 (67%)**	No benefit expected Relapsing: **10/32 (31%)** Sensitivity: **2/4 (50%)**	No benefit expected Relapsing: **19/53 (36%)** Sensitivity: **1/2 (50%)**	No benefit expected Relapsing: **16/220 (7%)** No cases treated	**Likely beneficial** Relapsing: **1/34 (3%)** Sensitivity: **17/22 (77%)**
G1269A	**Likely beneficial** Relapsing: **0/82 (0%)** Sensitivity: **2/3 (67%)**	Insufficient evidence Relapsing: 0/32 (0%) Sensitivity: 1/2 (50%)	**Likely beneficial** Relapsing: **0/53 (0%)** Sensitivity: **2/3 (67%)**	No benefit expected Relapsing: **34/220 (16%)** No cases treated	**Likely beneficial** Relapsing: **1/34 (3%)** Sensitivity: **5/5 (100%)**
L1196M **G1202R**	Insufficient evidence Relapsing: 2/82 (2%) No patients treated	Insufficient evidence Relapsing: 1/32 (3%) No patients treated	Insufficient evidence Relapsing: 0/53 (0%) No patients treated	Insufficient evidence Relapsing: 0/220 (0%) No patients treated	No benefit expected Relapsing: **4/34 (12%)** Sensitivity: **0/1 (0%)**

Clinical benefit
% of patients with only this mutation achieving clinical benefit or sensitivity on sequential treatment with this inhibitor

Relapsing
% of patients relapsing on this inhibitor harboring only this mutation

Three or more patients with treatment results

	>66%	33-66%	<33%
<5%	Likely beneficial	Possibly beneficial	No benefit expected
5-9%	Possibly beneficial	Conflicting evidence	No benefit expected
≥10%	Conflicting evidence	No benefit expected	No benefit expected

Two or less patients with treatment results

	100%	50%	0%	No patients treated
<5%	Possibly benefidial	Insufficient evidence	No benefit expected	Insufficient evidence
5-9%	Possibly beneficial	No benefit expected	No benefit expected	No benefit expected
≥10%	No benefit expected	No benefit expected	No benefit expected	No benefit expected

Figure 6 **Comparison of predicted clinical benefit predicted from each of the six *in vitro* models on drug sensitivity of *ALK* resistance mutations**

A cross-tabulation comparing observed clinical benefit based on tumor responses in patients to *in vitro* reports from literature on drug sensitivity of *ALK* mutational profiles (*Table ST1*). For each mutation-inhibitor combination, the predicted clinical benefit based on clinical treatment results (Figure 5 and *Table ST8*) is depicted at the top row. For these clinical results, green represents likely or possibly beneficial, red represents no benefit expected and gray represents insufficient clinical evidence (as presented in Figure 5). The six squares below represent (from left to right) the *in vitro* drug sensitivity as reported by preclinical studies (*Table ST2*). The annotation of these squares in the top row, G1123S, also applies to the other rows. These include reports by Fontana et al. ("F", first square, *EML4-ALK* V3),[23] Gainor et al. ("G", second square, *EML4-ALK* V1),[13] Yoda et al. ("Y1", third square, *EML4-ALK* V1; "Y3", fourth square, *EML4-ALK* V3),[24] and Horn et al. ("H1", fifth square, *EML4-ALK* V1; "H3", sixth square; *EML4-ALK* V3).[25] For these preclinical results, green corresponds to predicted sensitivity, red corresponds to predicted resistance and gray means no data was reported for this mutation-inhibitor combination.

Abbreviations: V1 = *EML4-ALK* fusion variant 1 (*EML4* exon 13 fused to *ALK* exon 20); V3 = *EML4-ALK* fusion variant 3 (*EML4* exon 6 fused to ALK exon 20);.

	Type of evidence	Alectinib	Brigatinib	Ceritinib	Crizotinib	Lorlatinib
G1123S	Clinical:	Possibly beneficial	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:	F G Y1 Y3 H1 H3	F G Y1 Y3 H1 H3	F G Y1 Y3 H1 H3	F G Y1 Y3 H1 H3	F G Y1 Y3 H1 H3
L1152R	Clinical:	Possibly beneficial	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:					
C1156Y	Clinical:	Likely beneficial	Possibly beneficial	Possibly beneficial	No benefit expected	Insufficient evidence
	In vitro:					
I1171N	Clinical:	No benefit expected	No benefit expected	Likely beneficial	Insufficient evidence	Possibly beneficial
	In vitro:					
I1171S	Clinical:	No benefit expected	Possibly beneficial	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:					
I1171T	Clinical:	Possibly beneficial	No benefit expected	Likely beneficial	Insufficient evidence	Insufficient evidence
	In vitro:					
F1174C	Clinical:	Possibly beneficial	Insufficient evidence	Possibly beneficial	No benefit expected	Possibly beneficial
	In vitro:					
F1174V	Clinical:	Possibly beneficial	Insufficient evidence	Insufficient evidence	Insufficient evidence	Possibly beneficial
	In vitro:					
V1180L	Clinical:	No benefit expected	Insufficient evidence	Possibly beneficial	Insufficient evidence	Possibly beneficial
	In vitro:					
L1196M	Clinical:	Conflicting evidence	No benefit expected	Possibly beneficial	No benefit expected	Likely beneficial
	In vitro:					
L1198F	Clinical:	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:					
G1202del	Clinical:	Possibly beneficial	Insufficient evidence	Insufficient evidence	Insufficient evidence	Possibly beneficial
	In vitro:					
G1202R	Clinical:	Conflicting evidence	No benefit expected	No benefit expected	No benefit expected	Likely beneficial
	In vitro:					
D1203N	Clinical:	Insufficient evidence	No benefit expected	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:					
S1206Y	Clinical:	Insufficient evidence	Insufficient evidence	Possibly beneficial	Insufficient evidence	Insufficient evidence
	In vitro:					
E1210K	Clinical:	Insufficient evidence	Possibly beneficial	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:					
F1245C	Clinical:	Insufficient evidence	Insufficient evidence	Possibly beneficial	Insufficient evidence	Insufficient evidence
	In vitro:					
G1269A	Clinical:	Likely beneficial	Insufficient evidence	Likely beneficial	No benefit expected	Likely beneficial
	In vitro:					
F1174C/D1203N	Clinical:	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence	Possibly beneficial
	In vitro:					
L1196M/L1198F	Clinical:	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:					
L1196M/G1202R	Clinical:	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence	No benefit expected
	In vitro:					
L1198F/G1202R	Clinical:	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence
	In vitro:					
D1203N/E1210K	Clinical:	Insufficient evidence	Insufficient evidence	Insufficient evidence	Insufficient evidence	Possibly beneficial
	In vitro:					

ison for these different sources of evidence. For each mutation-inhibitor combination, the top row depicts the predicted clinical benefit based on clinical treatment results, whereas the bottom row represents the *in vitro* drug sensitivity in preclinical studies. There was sufficient (non-conflicting) evidence of clinical benefit as well as *in vitro* evidence of response for 45 mutation-inhibitor combinations. Agreement between *in vitro* sensitivity and likelihood of clinical benefit ranged between 69% and 89%, ie, 89% for Yoda-V1 (8/9), 87% for Fontana-V3 (13/15), 83% for Gainor-V1 (29/35), 78% for Yoda-V3 (7/9), 69% for Horn-V3 (25/36) and 69% for Horn-V1 (24/35). Due to the major differences in *in-vitro* readouts of response methods, a direct statistical comparison between observed and predicted responses is not justified. Similarly, the large heterogeneity in the type of variants that are tested in the clinical models does not allow a head-to-head comparison to the determine the preclinical model with the highest predictive value.

Discussion

We have determined the likelihood of clinical benefit of ALK inhibitors in *ALK*-driven non-small cell lung carcinoma that developed distinct ALK-inhibitor induced on-target resistance mutations using data from 387 patients progressing after one or more lines of ALK inhibitors. Actionability of individual mutations was determined using published clinical reports, and compared to drug sensitivity derived from pre-clinical models. The ensuing summary of clinical benefit as presented in Figure 5 provides a good starting point for treating pulmonary oncologists and Molecular Tumor Boards for selecting the most optimal drug in individual patients.

Predicting Clinical Benefit From Sequential ALK Inhibition Using in vitro *Data and Patient Tumor Response*

The optimal choice of therapy varies for each resistance mutation, which is challenging in treatment decision-making. Although reports of *in vitro* drug sensitivity may be useful in choosing the optimal therapy, the various available models are limited by the fact that they only cover a portion of the full spectrum of possible *ALK* on-target resistance mutations, but for those that are comparable do not always agree. Moreover, the agreement for each model between *in vitro* prediction and observed clinical benefit ranged from 69% to 89%. Therefore, we recommend that treatment decisions should be based primarily on available clinical evidence of drug actionability, and resort to *in vitro* drug predictions in case of insufficient clinical evidence. Our overview on clinical evidence of drug actionability of *ALK* on-target resistance mutations may aid treating physicians and molecular tumor boards in choosing the most optimal sequential ALK inhibitor. Figure 5 summarizes the likelihood of clinical benefit for 5 available ALK inhibitors for the most frequently reported *ALK* resistance profiles.

There were 2 mutation-inhibitor combinations with conflicting clinical evidence: Alectinib-L1196M and Alectinib-G1202R. L1196M and G1202R mutations were highly prevalent as a resistance mechanism against alectinib (11% and 32%, respectively). However, patients who developed these mutations in response to a different inhibitor still demonstrated clinical benefit when treated with alectinib (9/11 L1196M-positive cases and 4 out of 6 G1202R-positive cases. Based on the currently available data, the explanation for this discrepancy remains elusive.

When considering treatment options for ALK inhibitor-resistant patients, some limitations should be considered. Firstly, these on-target resistance mutations remain rare events. The presented likelihood of clinical benefit represents the current understanding based on limited clinical reports of drug efficacy. Thus, the clinical evidence summarized in Figure 5 should be considered dynamic in time and will require revision when additional cases have been published.

Secondly, in case multiple mutations are detected, it is important to ascertain whether these mutations are present in the same subclone. Mutations detected *in cis* are present in a single clone, and the mutations should be assessed independent from the drug actionability of individual mutations. This is illustrated by L1196M and G1202R, which are individually sensitive to lorlatinib and thus confer sensitivity when detected in different subclones, but demonstrate high-level of resistance when present in the same subclone.[24] Selective ALK inhibitors targeting these lorlatinib-resistant compound mutations, such as NUV-655,[30] and TPX-0131,[31] are currently under development, but not yet available for use in patients. When mutations are detected *in trans*, they are more likely to represent multiple clones that may individually be sensitive as single mutants with the same inhibitor and may co-targeted with combination therapy. As shown previously, multiple mutations are more likely to be detected when genotyping plasma-derived circulating tumor DNA (ctDNA).[15,32] In this case, the different mutations are more likely to be derived from different clones, as ctDNA represents the full mutational load of all shedding progressive lesions whereas tissue samples represent a single lesion.

Finally, aside from *ALK* mutations, several other off-target mechanisms can cause acquired resistance to ALK inhibitors.[32] Examples include epithelial-to-mesenchymal transition (EMT),[33] small-cell lung cancer transformation,[34] *MET* amplification (which may sensitize tumors to crizotinib),[35] or *BRAF* p.(V600E).[36] These alternative resistance mechanisms need to be assessed in addition to *ALK* mutations to ascertain drug actionability of progressive lesions.

Factors Influencing the Distribution of ALK *on-target Resistance Mutations*

Each available ALK inhibitor demonstrated a distinct patterns of on-target resistance. These distributions were in line with previous reports.[13,15] Notably, whereas first- and second-generation ALK inhibitors mostly induced single *ALK* mutations, the majority (82%) of samples from patients pretreated with third-generation ALK inhibitor lorlatinib harbored more than one *ALK* mutation, with L1196M/G1202R (12%) as the most common mutational profile. This is known to occur more frequently with lorlatinib,[15] and suggests that tumors must accumulate different *ALK* mutations to acquire resistance to lorlatinib. This is also supported by the longer time it takes before disease progression occurs as compared to the other ALK inhibitors.[37] However, this accumulation of mutations by lorlatinib-resistant tumors in the current study may also be due to the fact that all patients in our study were pretreated with at least one first- or second-generation ALK inhibitor. The resistance mechanisms to first-line lorlatinib have not yet been inves-

tigated, but may be elucidated by post-hoc results of a recently published trial investigating the efficacy of first-line lorlatinib.[38]

Previous reports suggested that the distribution of on-target resistance mutations may also be affected by the *ALK* fusion variant.[39] Indeed, as reported by Lin et al., our analysis confirmed that L1196M was more prevalent in *EML4-ALK* V1 than in V3 (30% versus 5%; $P = .005$), after correction for previous ALK inhibitor. On the other hand, G1202R was more common in V3 than in V1 (24% vs. 4%; $P = .030$). Other commonly reported mutations were similarly distributed between both variants. Whereas a previous study has suggested that *EML4-ALK* V1 is associated with longer survival than V3 in crizotinib-treated patients,[40] differences in response or survival based on fusion variant could not be elucidated in the current study due to missing data in the majority of reported patients. Similarly, the influence of other *EML4-ALK* fusion variants or other fusion partners on mutation distribution or outcome could not be analyzed due to low frequencies of these variants in our pooled cohort.

Limitations of Methods Used in this Study

Our results were based on a pooled population of 387 patients treated in the UMCG and extracted from literature. Thirty-three patients (8.5%) were extracted from case reports. This may introduce a bias due to patients published in case reports having a naturally higher probability of exceptional response as opposed to those published in cohorts.

In addition, several papers included in our analysis were based on studies from the same dataset, such as those using the Massachusetts General Hospital (MGH) dataset. As a result, some patients were reported multiple times in different paper: for example, sample MGH087a has been reported on 4 separate occasions.[13,15,24,39] Although duplicates were excluded as much as possible, there is a risk that a few patients counted as unique are in fact duplicates.

It is good to realize that most patients in this literature-based cohort have been pretreated with crizotinib, and most patients treated with lorlatinib had been pretreated with more than one ALK inhibitor. The distribution of mutational profiles and potential clinical benefit will therefore change because after crizotinib now alectinib is first-line treatment and the next step is lorlatinib that may shift to the first-line, as is currently being tested in a phase III trial.[37]

Conclusions

ALK-driven non-small cell lung cancer patients that develop on-target resistance to ALK inhibitors benefit from treatment with sequential ALK inhibition. However, the choice of therapy depends strongly on the type of *ALK* mutation(s) detected at resistance. *In vitro* models to test ALK inhibitor actionability towards mutated *ALK* may support clinical decision making. However, in clinical practice, the number and diversity of mutations is higher than analyzed in the current pre-clinical models. Therefore, we recommend that treatment decisions should be based primarily on available clinical evidence of drug actionability, and resort to *in vitro* drug predictions in case of insufficient clinical evidence. Our overview on clinical evidence of drug actionability of on-target *ALK* resistance mutations (Figure 5) provides a good starting point for treating pulmonary oncologists and Molecular Tumor Boards for selecting the most optimal drug in individual patients. These results support testing for on-target *ALK* resistance mutations in *ALK* fusion-positive NSCLC patients progressing on an ALK inhibitor to allow personalized treatment with additional ALK inhibitors.

Clinical Practice Points

- *ALK* fusion-positive non-small-cell lung cancer patients treated with ALK inhibitors frequently develop on-target resistance.
- Clinical evidence for the subsequent line of treatment with currently available ALK inhibitors have been presented as case reports, case series and preclinical models.
- This comprehensive review summarizes the clinical evidence of the actionability of *ALK* on-target resistance mechanisms with ALK inhibitors
- The clinical mutation-drug response matrix may serve as a practical guide to select the most optimal ALK inhibitor for patients with on-target resistance to first line ALK inhibitor.

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Disclosures

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cllc.2021.06.011.