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## Prospective Multicenter Validation of the Detection of ALK Rearrangements of Circulating Tumor Cells for Noninvasive Longitudinal Management of Patients With Advanced NSCLC

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1 **Prospective multi-center validation of the detection of *ALK* rearrangements of**  
2 **circulating tumor cells for non-invasive longitudinal management of patients**  
3 **with advanced non-small cell lung cancer**  
4

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91

92 **ABSTRACT**

93 **INTRODUCTION:** Patients with advanced-stage non-small cell lung cancer (NSCLC)  
94 whose tumors harbor an anaplastic lymphoma kinase (*ALK*) gene rearrangement  
95 benefit from treatment with multiple ALK inhibitors (ALKi). Approximately 30% of  
96 tumor biopsies contain insufficient tissue for successful *ALK* molecular  
97 characterization. This study evaluated the added value of analyzing circulating tumor  
98 cells (CTCs) as a surrogate to *ALK* tissue analysis and as a function of the response  
99 to ALKi.

100 **PATIENTS AND METHODS:** We conducted a multi-center prospective observational  
101 study (NCT02372448) of 203 patients with stage IIIB/IV NSCLC across 9 French  
102 centers, 81 were *ALK+* (IHC or FISH) and 122 *ALK-* on paraffin embedded tissue  
103 specimens. Blood samples were collected at baseline and at 6 and 12 weeks after  
104 ALKi initiation or at disease progression. *ALK* gene rearrangement was evaluated  
105 with CTCs using immunocytochemistry (ICC) and FISH analysis after enrichment  
106 using a filtration method.

107 **RESULTS:** At baseline, there was a high concordance between the detection of an  
108 *ALK* rearrangement in tumor tissue and in CTCs as determined by ICC (sensitivity,  
109 94.4%; specificity 89.4%). The performance was lower for the FISH analysis  
110 (sensitivity, 35.6%; specificity, 56.9%). No significant association between baseline  
111 levels or the dynamic change of CTCs and survival was observed in *ALK*-positive  
112 patients.

113 **CONCLUSIONS:** CTCs can be used as a complementary tool to a tissue biopsy for  
114 the detection of *ALK* rearrangements. Longitudinal analysis of CTCs showed promise  
115 for real-time patient monitoring and improved delivery of molecularly guided therapy  
116 in this population.

117

118 **Keywords:** *ALK*, CTCs, immunocytochemistry, FISH, lung adenocarcinoma.

119

## 120 **INTRODUCTION**

121 Non-small cell lung cancer (NSCLC) harboring *ALK* receptor tyrosine kinase (*ALK*)–  
122 rearrangements is a distinct subset of lung cancers, occurring in approximately 4% of  
123 patients at an advanced stage.<sup>1</sup> Several *ALK* inhibitors (*ALKi*) have been approved  
124 by the U.S. Food and Drug Administration and the European Medicines Agency for  
125 the treatment of patients with *ALK*-positive NSCLC at first or latter lines of  
126 treatment.<sup>2, 3</sup>

127 The eligibility of NSCLC patients to receive *ALKi* relies on a companion  
128 immunohistochemistry (IHC) performed on tumor tissue<sup>4</sup>, e.g. IHC or FISH that need  
129 a sufficient number of tumor cells to be conclusive. However, procedures providing  
130 small biopsy samples (e.g. fine needle aspiration, transthoracic needle biopsy,  
131 endobronchial ultrasound, bronchoscopy, bronchial biopsies) may lack a sufficient  
132 number of tumor cells. Furthermore, invasive procedures can be related to a relative  
133 risk of morbidity. Moreover, because tumors evolve and adapt to targeted therapies,  
134 it is becoming increasingly important to monitor the molecular changes occurring in  
135 NSCLC patients in “real-time” especially when selecting subsequent lines of  
136 treatment.<sup>5</sup> In addition, the degree of benefit from *ALKi* varies widely between  
137 NSCLC patients, despite sharing molecular drivers. Longitudinal biopsies upon  
138 disease progression have been useful in identifying the molecular mechanisms  
139 driving resistance to *ALKi*. Yet, repeated tissue biopsies are not always feasible and  
140 sampling of a single tumor site may not reflect the spatial heterogeneity of resistance  
141 mechanisms.<sup>6</sup>

142 In daily practice, accurate identification of *ALK* fusions as well as tumor evolution  
143 during therapy could have significant impact on the therapeutic strategy likely to have  
144 the greatest impact on patient outcome.<sup>6</sup> Therefore, developing non-invasive  
145 surrogate methods suitable to detect *ALK* rearrangements at baseline and during  
146 tumor evolution can be pivotal to improving the success rate of *ALKi*.

147 It is now well admitted that liquid biopsy (LB) is a simple, non-invasive and easily  
148 repeatable alternative to tumor biopsies to assess the tumor’s molecular status and  
149 to monitor genetic changes in “real-time”.<sup>7</sup> Among the LB components, circulating  
150 tumor cells (CTCs) contain tumorigenic cell clones with high relevance for metastatic  
151 dissemination.<sup>8</sup> CTCs are likely released from distinct metastatic sites and may  
152 inform on the genomic heterogenic picture of the metastatic disease. In contrast to  
153 tumor biopsies, CTCs could allow longitudinal monitoring at different time-points

154 during treatment, thereby having the potential to guide therapeutic decisions in “real-  
155 time”.<sup>9</sup>

156 We and other groups have reported the feasibility of *ALK*-rearrangement detection  
157 with CTCs collected from patients with *ALK*-positive NSCLC at baseline by using a  
158 filter-based technique for CTC enrichment and *ALK* testing by FISH and/or  
159 immunocytochemistry (ICC).<sup>10-12</sup> To confirm these previous retrospective feasibility  
160 studies, we performed the first multi-center prospective clinical validation of the use  
161 of CTCs as a surrogate for tumor tissue for *ALK* testing, thus aiding in  
162 prognostication and therapy selection at baseline and during treatment with ALKi of  
163 patients with stage IIIB/IV NSCLC.

164

## 165 **MATERIALS AND METHODS**

### 166 **Patients and samples**

167 This multi-center prospective observational study (NCT02372448) was conducted  
168 across 9 French clinical institutions from January 23, 2015 through November 21,  
169 2019 in accordance with the Declaration of Helsinki and the European Directive and  
170 Good Clinical Practices. The study was approved by the Ethics Committee (CPP  
171 Sud-Méditerranée V) and authorized by the National Security Agency for Medicines  
172 and Health Products (ANSM). Informed-written consent was obtained from all  
173 patients. Eligible patients had histologically confirmed stage IIIB/IV NSCLC (**Table 1**).  
174 A few number of patients had initially diagnosed stage I-III A NSCLC, enrolled in the  
175 study at the time of the metastatic relapse (**Table 1**). For these patients, a re-biopsy  
176 was performed to evaluate the *ALK* status. These patients may have had prior  
177 chemotherapy delivered as part of their primary treatment but must have completed  
178 primary therapy at least 3 months before their entry in the study.

179 The pre-specified primary outcome was sensitivity and specificity of FISH between  
180 tumor tissue and CTCs at baseline as well as a follow-up « visit 1 » after six weeks  
181 (V1) and follow-up « visit 2 » after 12 weeks (V2). Pre-specified secondary outcomes  
182 included sensitivity and specificity of ICC at Baseline, V1 and V2 as well as  
183 association of *ALK* positive CTCs and clinical outcome.

184 *ALK* rearrangement was locally tested on tumor tissue by IHC (anti-*ALK* antibody,  
185 pre-diluted, clone D5F3, Roche Diagnostics, Basel, Switzerland) and FISH (Vysis  
186 *ALK* Break Apart Rearrangement Probe Kit; Abott Molecular Inc., Des Plaines, USA),



187 performed according to pre-specified standard operating procedures in ISO 15189  
188 accredited laboratories (Accreditation n° 8-3034;  
189 <https://tools.cofrac.fr/annexes/sect8/8-3034.pdf>).

190 Thirty ml of peripheral blood was collected in EDTA or Streck tubes at baseline (V0)  
191 and at 6 (V1) and 12 (V2) weeks on ALKi initiation and/or at disease progression.<sup>13</sup>  
192 CTCs were enumerated on ISET® filters (Isolation by Size of Tumor Cells, Rarecells,  
193 Paris, France), as previously reported.<sup>10, 14</sup> Briefly, stained filters were examined by a  
194 senior thoracic cytopathologist using light microscopy in two steps: (i) screening at ×  
195 20 magnification to perform cells localization and detection, then, (ii) observation at ×  
196 100 magnification using an oil immersion for detailed cytomorphological analysis.  
197 CTCs were defined as cells presenting all the following criteria: (i) nuclear size equal  
198 or larger than one pore (i.e., equal or larger than 8 μm in diameter); (ii) irregularity of  
199 the nuclear contour; (iii) presence of a well defined and visible cytoplasm; and, (iv)  
200 high nuclear-to-cytoplasmic ratio (>0.8).

201 Consequently, CT scans and/or MRIs were performed at baseline and during follow-  
202 up. The response to ALKi (**Supplementary Table 1**) was evaluated according to the  
203 “Response Evaluation Criteria in Solid Tumors” (RECIST) version 1.1. The disease  
204 control rate was defined as a complete or partial response or stable disease based  
205 on RECIST.

206

### 207 **ALK immunocytochemistry and FISH on ISET-enriched CTCs**

208 All ISET filters were sent to the coordinating team at the CHU Nice for centralized  
209 ALK analysis. ICC and FISH were performed on CTCs isolated using the ISET  
210 method on unstained spots of the corresponding filters containing CTCs detected on  
211 four spots colored with May-Grünwald-Giemsa [15]. Three spots were used for ICC  
212 and three spots were used for FISH per filter. For ICC, the spots were incubated with  
213 a primary antibody against the ALK protein (pre-diluted, clone D5F3; Ventana) for 32  
214 min at room temperature. FISH analysis was carried out using a break-apart probe  
215 for the ALK gene (Vysis ALK Break Apart Rearrangement Probe Kit, Abbott  
216 Molecular Inc.) in accordance with the manufacturer’s instructions. Cells showing at  
217 least one split signal or single 3’ signals were considered positive for an *ALK*  
218 rearrangement. ISET filters were examined independently and blinded to clinical,  
219 IHC, ICC, FISH data and tissue genotype. We used the human NSCLC cell line

220 H2228 obtained from ATCC (Manassas, VA, USA) as an *ALK* rearrangement positive  
221 control in every run.<sup>10</sup>

222

### 223 **Statistical analysis**

224 The performance of *ALK* assessment on ICC or FISH was compared to the paired  
225 tumor biopsy and equivalent method using sensitivity and specificity analysis. The  
226 same analysis was carried out first on the population with available CTCs (n=185  
227 patients and n=97 analyzed for ICC and FISH respectively) and second on the entire  
228 population including patients with no available CTCs (n=203 patients analyzed). In  
229 this latter case, *ALK* assessment was not available by using ICC or FISH methods,  
230 we used the worst scenario by considering all *ALK* positive cases on the biopsy as  
231 false negative with ICC and FISH and all *ALK* negative on the biopsy as false  
232 positive with ICC and FISH.

233 The association to clinical variables was assessed using  $\chi^2$ , Student-*t* test and  
234 Fisher's exact test analyses.

235 Progression-free survival (PFS) was defined as the time between the date of the start  
236 of *ALK*i and the date of clinical or radiological progression according to RECIST, or  
237 death. Overall survival (OS) was defined as the time between the date of the start of  
238 *ALK*i and the date of death. Patients who were progression-free and alive at the time  
239 of analysis were censored.

240 All statistical analyses were performed using R (version 3.5.0, R Foundation for  
241 Statistical Computing) or Python (version 3.7.2, Python Software Foundation).

242

## 243 **RESULTS**

### 244 **Patient characteristics**

245 Peripheral blood was collected from 193 patients with stage IIIB/IV NSCLC and 10  
246 patients with initially diagnosed stage I-III A NSCLC enrolled in the study at the time  
247 of the metastatic relapse (**Table 1**). In total, 81 (40%) patients with *ALK*-positive  
248 NSCLC and 122 (60%) patients with *ALK*-negative NSCLC were included in the  
249 study cohort. Most *ALK*-positive patients were never or former smokers (97%) with  
250 adenocarcinoma tumor histology (99%).

251 At baseline (V0), CTCs were detected in 187 (92%) patients (range, 2–621/4 ml;  
252 median, 62 CTCs/4 ml; **Fig. 1A**). A significantly higher number of CTCs were  
253 detected in *ALK*-negative patients than in *ALK*-positive patients (median CTCs = 71

254 vs. 21;  $P = 0.0098$ ) (**Fig. 1A**). CTCs were not detected in 16/203 (8%) patients,  
255 10/203 (5%) *ALK*-positive patients and 6/203 (3%) *ALK*-negative patients.

256 At first visit (V1) during monitoring of *ALK*-positive patients, 27/81 (33%) patients had  
257 CTCs (range, 6–177/ 4 ml; median, 6 CTCs/ 4 ml; **Fig. 1B**). At the second follow-up  
258 visit (V2), we detected CTCs (range, 11–173/ 4 ml; median, 19 CTCs/ 4 ml) in 17/81  
259 (21%) *ALK*-positive patients (**Fig. 1B**).

260  
261

### 262 **Performance of CTCs for detection of *ALK* rearrangements**

263 At baseline, there was substantial concordance between the detection of an *ALK*  
264 rearrangement in the tumor tissue (IHC and FISH) and CTCs (**Fig. 2**). The sensitivity  
265 and the specificity for ICC were 94.36% and 89.39%, across all samples, and 100%,  
266 respectively, for patients with detectable CTCs at baseline. Lower concordance was  
267 observed for FISH with 35.63% sensitivity and 56.89% specificity, across all samples,  
268 and 100%, respectively, for patients with detectable CTCs at baseline.

269 Moreover, the levels of CTCs or *ALK*-positive CTCs significantly decreased with ALKi  
270 treatment (**Fig. 1B; Supplemental Fig. S1**).

271 In all positive samples by FISH and IHC, 100% of detected CTCs were positive in  
272 ICC with an intensity score of 3. All but one FISH positive sample were 100% positive  
273 for rearranged *ALK* with one sample at baseline having only 80% FISH positive  
274 cells).

275

### 276 **Association between *ALK*-CTCs and clinical parameters or outcome**

277 An exploratory analysis revealed a significant association between the status of *ALK*-  
278 CTCs, younger age and non-smoking status. *ALK*-positive CTCs were present more  
279 often in younger patients (mean, 60 vs. 64 years,  $P=0.038$ ) and non-smokers  
280 ( $P<0.0001$ ). All the other clinical parameters were not significantly associated with  
281 *ALK*-rearranged CTC counts at baseline. In addition, in *ALK*-positive patients, the  
282 levels of CTCs significantly correlated to age and stage (**Table 2**).

283 At the first follow-up visit, 25 (31%) patients had disease progression according to  
284 RECIST.

285 For the univariate analysis, the level of CTCs at baseline was assigned into low and  
286 high groups according to their respective median in *ALK*-positive patients. We did not

287 observe a significant association between baseline CTCs levels and OS ( $P=0.244$ ,  
288 HR=0.59, 95% CI, 0.24–1.5) or PFS ( $P=0.591$ , HR=0.84, 95% CI, 0.44–1.6; **Fig. 3**).  
289 In addition, we considered the dynamic change in the CTC counts under treatment  
290 as a possible parameter to evaluate. Patients were assigned into two groups: 13  
291 patients presented stable or increased numbers of *ALK*-CTCs (median increase, 36;  
292 range, 2–290), whereas 37 patients presented a decrease in *ALK*-CTCs (median  
293 decrease, –7; range, –250 to –280). However, we did not observe a statistically  
294 significant association between the dynamic change in the CTC number on *ALKi* and  
295 response to treatment with a univariate analysis ( $P=0.268$ ; **Fig. 4, Supplemental**  
296 **Fig. S2, and Supplemental Fig. S3**).

## 297 **DISCUSSION**

298 In this study, we demonstrated that CTCs could be a reliable surrogate to a  
299 tissue biopsy for the detection of *ALK* rearrangements in advanced-stage or recurrent  
300 NSCLC patients. Moreover, the decrease in the levels of *ALK*-positive CTCs could be  
301 highly informative of the predictive efficacy of *ALKi* in *ALK*-rearranged NSCLC  
302 patients. Our study offers a promising perspective into the use of CTCs for real-time  
303 monitoring of this population.

304 Currently, the eligibility of NSCLC patients for *ALKi* relies on an assay performed  
305 on a tumor sample collected by biopsy.<sup>15</sup> This invasive procedure is associated with  
306 a non-negligible risk of morbidity.<sup>16</sup> The detection of an *ALK* rearrangement is  
307 currently performed on small biopsies or fine-needle aspirates and can be hindered  
308 by the limited quantity of tissue and/or the number of tumor cells. Tumor tissue is  
309 difficult to obtain from patients with advanced/metastatic lung cancer for whom  
310 surgery is rarely a component of the treatment care. Thus, a subset of NSCLC  
311 patients can be deprived of potentially more efficient therapies. Finding alternative  
312 and more effective means of diagnosing and monitoring *ALK* rearrangements are  
313 critical issues to identify the patients who may benefit from treatment with *ALKi*. For  
314 instance, detection of *ALK* fusions in circulating tumor DNA (ctDNA) has been  
315 previously tested using next-generation sequencing (NGS-, RT-PCR or RNA-Seq  
316 with variable sensitivities (70%-80%) for the different assays.<sup>17, 18</sup> Thus, in contrast to  
317 *EGFR* mutations, detection of *ALK* rearrangements with ctDNA is rarely implemented  
318 in daily practice.

319 Recently, the prospective multi-center NILE study demonstrated that ctDNA can  
320 rescue biomarker-positive patients with non-diagnostic tissue results, suggesting the

321 clinical utility of ctDNA in newly diagnosed metastatic NSCLC.<sup>19</sup> However, the LB  
322 test used was compared to a standard-of-care tissue genotyping test and not the  
323 tissue-based NGS, and the results were only applicable to the Guardant360 test and  
324 not to other LB tests.<sup>19</sup> In addition, the FoundationOne@Liquid CDx assay, a pan-  
325 cancer ctDNA-based comprehensive genomic profiling assay, was recently approved  
326 by the FDA.<sup>20</sup> However, the number of cases with an *ALK* rearrangement used for  
327 the analytic validation was quite low.<sup>20</sup>

328 A "LB" of CTCs present in the blood and the characterization of their *ALK* status  
329 is an appealing alternative that meets the urgent needs of these patients. In addition,  
330 no CTC-based molecular test is currently available in routine clinical practice.<sup>21</sup>

331 In this setting, CTCs which have been shown to be detectable by ISET in 80%  
332 to 100 % of late stages lung cancer patients represent a non-invasive and easily  
333 accessible source of tumor material for assessing *ALK* rearrangement in a dynamic  
334 manner.<sup>14, 22</sup> In our study, we did not isolate CTCs in only 8% of patients, which is in  
335 the range of sensitivity described for the detection of CTCs by ISET approach in  
336 NSCLC patients.<sup>14, 22-24</sup> The lack of CTC detection in these patients could be related  
337 to either technical or biological hurdles. It may be that patients truly present negative  
338 for CTCs, or CTCs may be lost at different times during the filtration and staining  
339 process, (i) through the pores of 8  $\mu$ m during the procedure of filtration, and/or (ii)  
340 during the sequential washes used during the staining procedure performed after  
341 filtration.<sup>23, 25</sup> The high sensitivity and specificity of ISET were two essential starting  
342 points for the feasibility of the present independent and multi-center project.<sup>10</sup> In our  
343 first feasibility mono-centric study, we showed excellent concordance for the *ALK*  
344 status between CTCs and the matched tumor tissue sample in a limited series of 87  
345 patients with advanced NSCLC, with only 5 patients harboring an *ALK*  
346 rearrangement.<sup>10</sup>

347 In this multi-center prospective observational study across 9 centers there was  
348 perfect concordance (100%) between the tissue and CTC *ALK* analysis, in all cases  
349 with CTCs isolated on filters (92%). In the small proportion of cases without CTCs,  
350 the *ALK* status could not be determined on the filters, suggesting technical  
351 challenges rather than biological heterogeneity, as described previously.<sup>23</sup>

352 Our study presents some limitations. One ISET spot contains on average  
353 5,000 white blood cells, mainly leukocytes, and only a small number of CTCs.<sup>26</sup>

354 Consequently, the manual analysis of one ISET spot to identify *ALK*-rearranged  
355 CTCs by FISH is laborious, time consuming and operator-dependent.

356 In this setting, the *ALK*-ICC or FISH analysis increases the feasibility,  
357 precision and reliability of *ALK* testing of filtration-enriched CTCs and may contribute  
358 to the validation and qualification of CTC assays for *ALK*-rearranged NSCLC  
359 patients.

360 It is worth noting that other non-invasive biological components that can be  
361 isolated from peripheral blood, such as ctDNA, circulating exosomes, platelet RNA or  
362 ctRNA, are currently being investigated for the detection of gene rearrangements.<sup>18,</sup>  
363 <sup>27, 28</sup>, although the sensitivity and precision of these strategies needs to be further  
364 evaluated and compared with those of CTC assays.

365 Whereas each of these approaches has the potential to provide novel  
366 diagnostic information and their exploration is highly encouraged, ctDNA certainly  
367 represents the most mature example of the investigation of the LB in clinical practice  
368 for NSCLC patients, in particular for gene mutation detection.<sup>5, 6, 29, 30</sup>

369 However, the analysis of ctDNA requires extensive deep sequencing of  
370 genomic DNA for reliable detection of the chromosomal break-point.<sup>31</sup> Moreover,  
371 digital-droplet PCR, BEAMing and NGS have been reported to have a promising  
372 sensitivity for the detection of *ALK* gene fusions, but are more variable than that  
373 observed for point mutations and indels.<sup>32</sup> Further studies should focus on orthogonal  
374 comparisons using CTCs, ctDNA and ctRNA to better characterize the presence of  
375 *ALK* rearrangements and to validate the clinical utility of detecting these genomic  
376 variants in plasma, notably in naïve treated patients with no tumor tissue  
377 accessibility.

378 In conclusion, this multi-center study is unique as no CTC-based *ALK*  
379 rearrangement assay has yet been independently validated with clinical samples.  
380 The development of a non-invasive predictive test through the genomic analysis of  
381 CTCs is a clinically relevant goal for non-invasive stratification of cancer patients,  
382 avoiding morbidity related to lung biopsy and surgery. Analysis with a blood sample  
383 should allow patient eligibility to targeted therapies to be determined. Small-sized  
384 samples with a low percentage of tumor cells and poor biopsy quality from NSCLC  
385 patients can often jeopardize optimal treatment management; CTC-based *ALK*  
386 testing can be useful to guide the choice of anti-*ALK* targeted therapy.

387

388

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393

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414 work.

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416 outside the submitted work.

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418 Squibb, Pfizer, Novartis, Bayer, Merck, MSD, Qiagen, Illumina, ThermoFisher  
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420 All other authors have no conflict of interest to declare.

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518 **Legend to Tables**

519

520 **Table 1.** Main clinicopathological data of the 203 NSCLC patients included in this  
521 study.

522 **Table 2.** Correlation analysis between *ALK*-positive patients with detectable CTCs  
523 and *ALK*-positive patients without detectable CTCs.

524

525

526 **Legend to Figures**

527 **Figure 1.** Boxplots showing the levels of CTCs according to **(A)** the *ALK* status at  
528 baseline « visit » 0 (V0), and **(B)** during the monitoring of *ALK*-positive patients at  
529 follow-up « visit 1 » after six weeks (V1) and follow-up « visit 2 » after 12 weeks (V2).

530

531 **Figure 2.** Representative images of *ALK*-positive **(A-C)** and *ALK*-negative **(D-F)**  
532 NSCLC cases.

533 **A.** Circulating tumor cells showing malignant cyto-morphological criteria isolated by  
534 the ISET method (original magnification  $\times 200$ ; MGG staining; bar: 20  $\mu\text{m}$ ).

535 **B.** Circulating tumor cells showing an intense and cytoplasmic staining (score 3+)  
536 (*ALK* immunostaining using D5F3 mAb, immunoperoxidase; original magnification  
537  $\times 200$ ; bar: 20  $\mu\text{m}$ ).

538 **C.** Circulating tumor cell nuclei hybridized with the Vysis *ALK* Break Apart  
539 Rearrangement Probe Kit. The two probes (3' red; 5' green) show a distinct  
540 separation of the red and green signals (arrows) indicating a rearrangement in the  
541 2p23 *ALK*-gene locus (original magnification  $\times 1000$ ; bar: 20  $\mu\text{m}$ ).

542 **D.** Circulating cells showing malignant cytomorphological criteria isolated by the ISET  
543 method (original magnification  $\times 200$ ; MGG staining; bar: 20  $\mu\text{m}$ ).

544 **E.** Circulating tumor cells showing lack of staining (score 0) (D5F3 mAb,  
545 immunoperoxidase; original magnification  $\times 200$ ; bar: 20  $\mu\text{m}$ ).

546 **F.** Circulating tumor cell nuclei hybridized with the Vysis *ALK* Break Apart  
547 Rearrangement Probe Kit. The probes give overlapping signals in nuclei without the  
548 rearrangement (arrows; original magnification  $\times 1000$ ; bar: 20  $\mu\text{m}$ ).

549

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551

552 **Figure 3.** Kaplan-Meier survival curves according to the median of CTCs level (n=62  
553 CTCs/4 ml) at baseline from the 81 NSCLC *ALK*-positive patients. **A)** Overall  
554 survival, and **B)** Progression-free survival curves. The *P*-values were calculated  
555 using the Log-rank test.

556

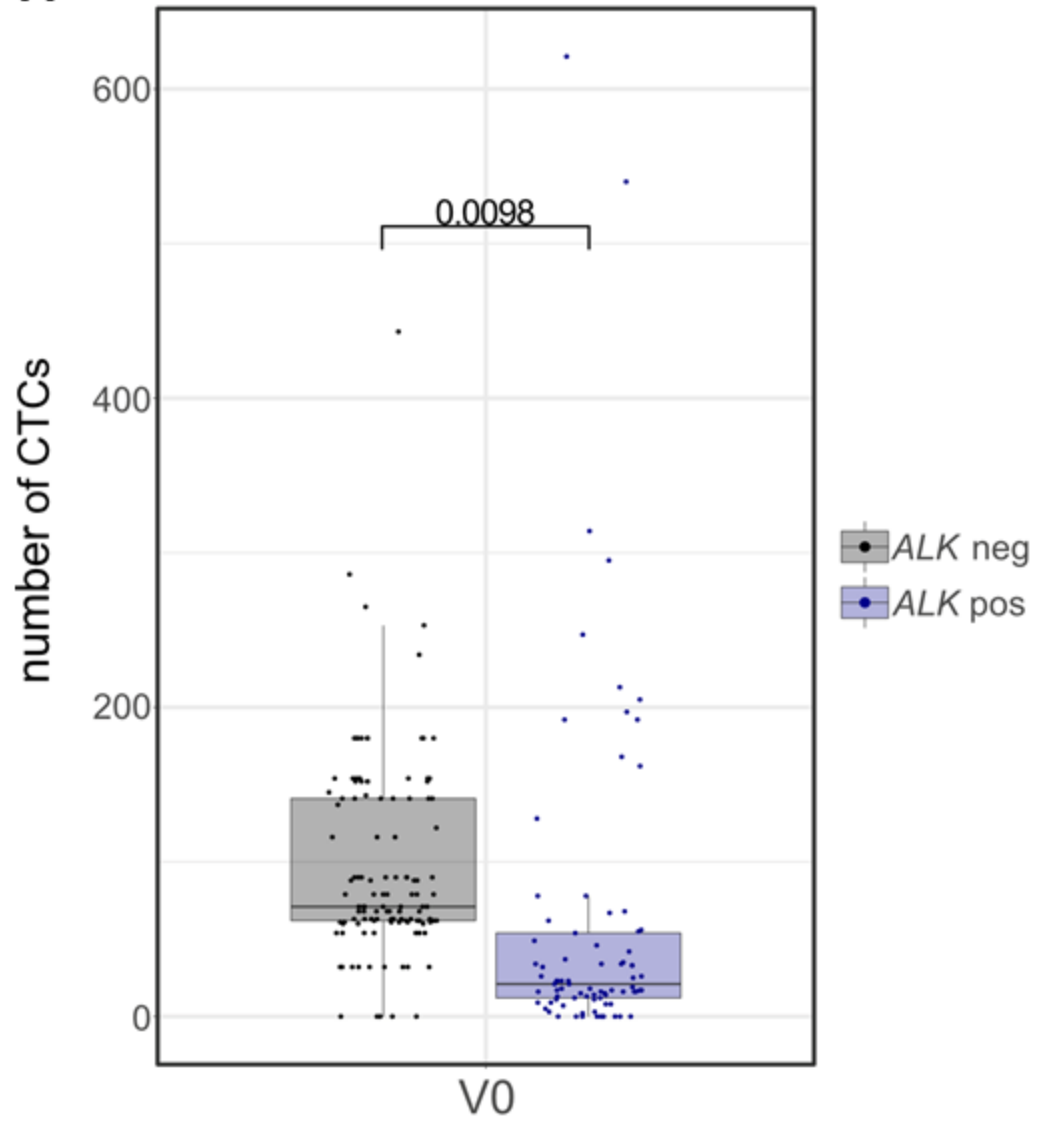
557 **Figure 4. Plots of *ALK*-CTC counts per 3 ml of blood based on ICC detection in**  
558 **patients who were serially sampled (e.g., paired time points for the same**  
559 **patient) during treatment with *ALK* inhibitors. A)** “Spaghetti” plots depicting the  
560 *ALK*-CTC counts in relation to PD, SD, and PR/CR on separate panels. **B)** Box-plots  
561 depicting *ALK*-CTC changes in relation to CR, PR, SD, PD from baseline to visit 1,  
562 and from visit 1 to visit 2.

563 *The color of the lines is highlighting the respective TKI used at baseline for the*  
564 *treatment of each patient. The dots highlight the response according to RECIST v1.1*  
565 *at the two respective follow up visits (Visit 1 and Visit 2). Only patients with an *ALK*-*  
566 *positive tumor are included in the analysis.*

567

Figure 1

**A**



**B**

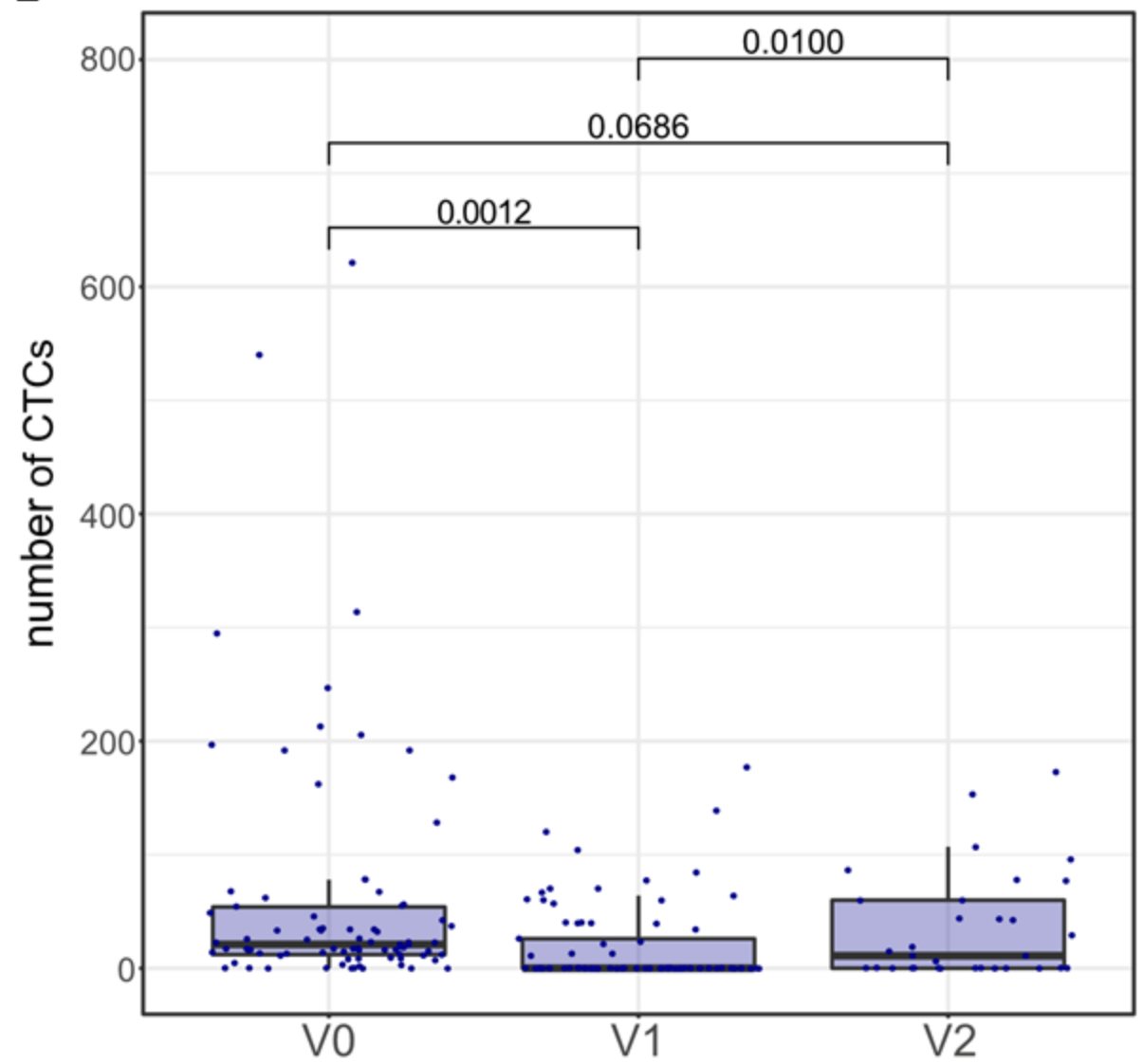
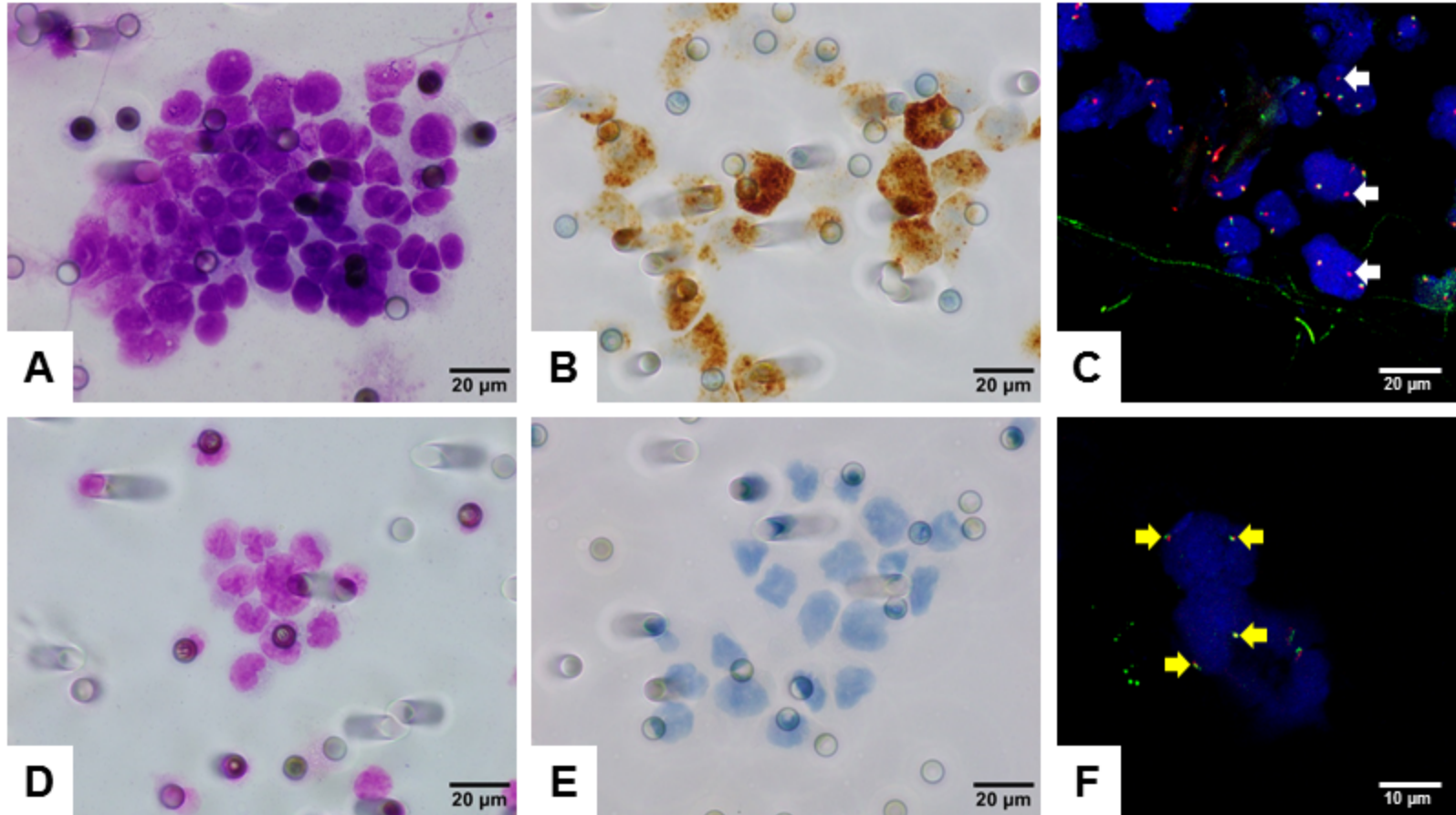
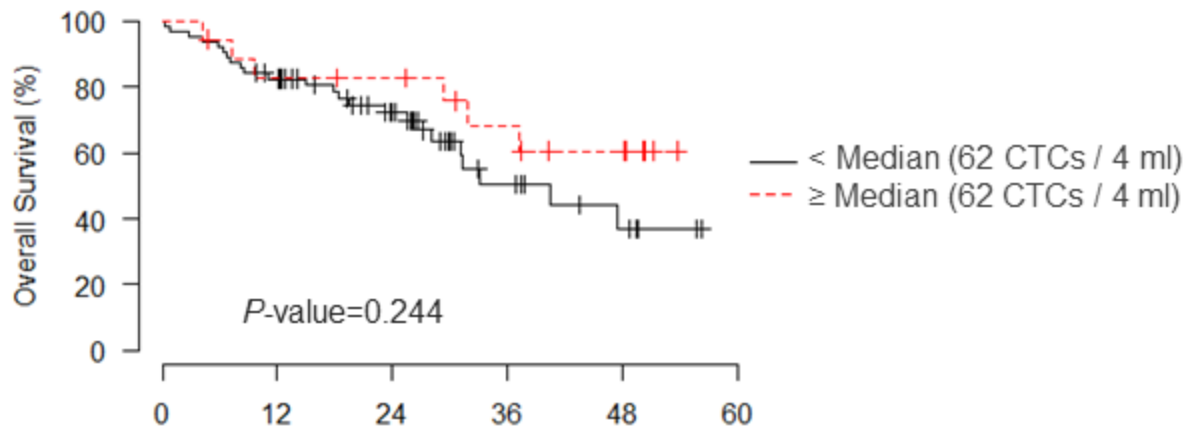


Figure 2



### Figure 3

**A**

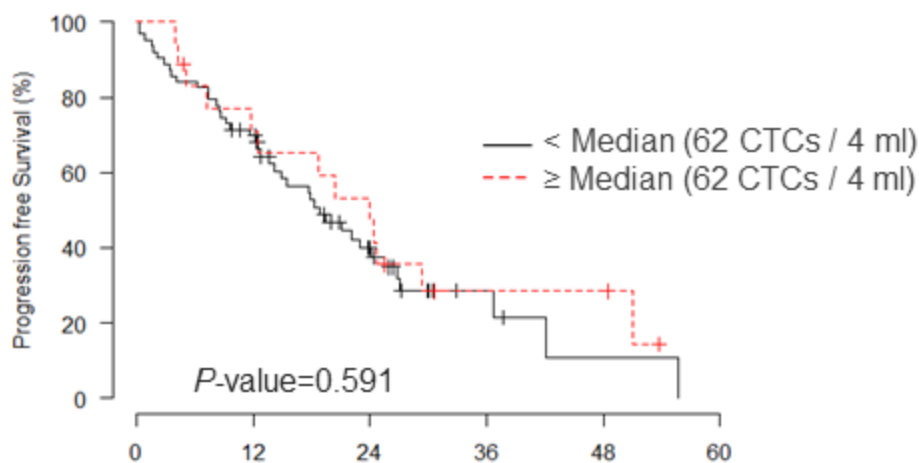


< Median		Months				
Time (months)	0	12	24	36	48	60
Patients at risk	83	50	30	11	5	
Number of events	0	11	5	6	2	
Censored patients	0	2	15	13	4	
Survival rate	1	0.82	0.72	0.51	0.37	
Lower limit CI 95%	1	0.74	0.62	0.38	0.21	
Upper limit CI 95%	1	0.92	0.85	0.71	0.65	

≥ Median		Months				
Time (months)	0	12	24	36	48	60
Patients at risk	18	14	13	9	6	
Number of events	0	3	0	2	1	
Censored patients	0	1	1	2	2	
Survival rate	1	0.83	0.83	0.68	0.61	
Lower limit CI 95%	1	0.67	0.67	0.48	0.4	
Upper limit CI 95%	1	1	1	0.96	0.92	

**B**

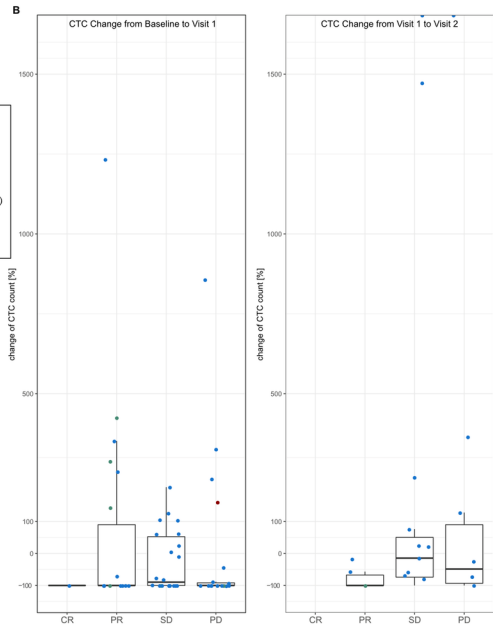
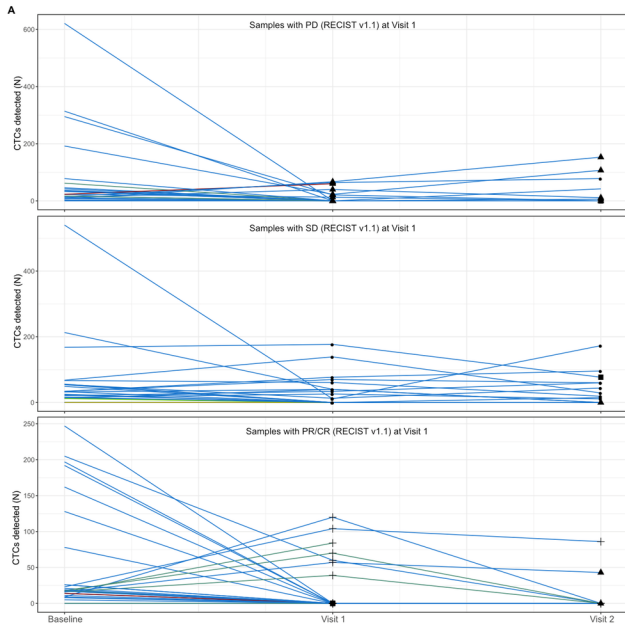


< Median		Months				
Time (months)	0	12	24	36	48	60
Patients at risk	83	42	18	4	1	
Number of events	0	19	15	4	2	
Censored patients	0	2	11	8	1	
Survival rate	1	0.7	0.4	0.29	0.11	
Lower limit CI 95%	1	0.59	0.29	0.18	0.02	
Upper limit CI 95%	1	0.82	0.58	0.46	0.51	

≥ Median		Months				
Time (months)	0	12	24	36	48	60
Patients at risk	18	12	8	3	3	
Number of events	0	5	4	3	0	
Censored patients	0	1	0	2	0	
Survival rate	1	0.71	0.47	0.28	0.28	
Lower limit CI 95%	1	0.53	0.29	0.13	0.13	
Upper limit CI 95%	1	0.96	0.78	0.62	0.62	

# Figure 4





**Table 1**

	<b>ALK – n (%)</b>	<b>ALK + n (%)</b>
<b>Overall</b>	122 (60%)	81 (40%)
<b>Age (median, range)</b>	64 (33-92)	62 (29-82)
<b>Gender</b>		
Male	85 (70%)	39 (48%)
Female	37 (30%)	42 (52%)
<b>Smoking status</b>		
Current smoker	49 (40%)	6 (7%)
Former smoker	65 (53%)	30 (37%)
Never smoker	8 (7%)	45 (56%)
<b>Histology</b>		
Adenocarcinoma	98 (80%)	80 (99%)
Squamous cell carcinoma	15 (12%)	0 (0%)
NSCLC NOS	9 (8%)	1 (1%)
<b>Disease status at study inclusion</b>		
Metastatic relapse <sup>#</sup>	1 (1%)	9 (11%)
Stage IIIB	17 (14%)	10 (12%)
Stage IV	104 (85%)	62 (77%)
<sup>#</sup> Cases with initially diagnosed stage I-IIIa NSCLC with metastatic relapse and rebiopsy to evaluate the <i>ALK</i> status.		

**Table 2**

	<b>ALK + CTCs &lt; median n (%)</b>	<b>ALK + CTCs ≥ median n (%)</b>	<b>P-value</b>
<b>Overall</b>	63 (77.7%)	18 (22.3%)	
<b>Age (mean, SD)</b>	61.6 (13.3)	52.3 (12.2)	0.009
<b>Gender</b>			0.4305
Male	32 (50.8%)	7 (38.9%)	
Female	31 (49.2%)	11 (61.1%)	
<b>Smoking status</b>			0.749
Current smoker	4 (6.4%)	2 (11.2%)	
Former smoker	25 (39.7%)	7 (38.8%)	
Never smoker	34 (53.9%)	9 (50%)	
<b>Histology</b>			1
Adenocarcinoma	61 (96.8%)	18 (100%)	
Squamous cell carcinoma	1 (1.6%)	0 (0%)	
NSCLC NOS	1 (1.6%)	0 (0%)	
<b>Stage</b>			0.02
Stage I / II	2 (3.2%)	4 (22.2%)	
III / IV	61 (96.8%)	14 (77.8%)	