



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

Marius Ilié, MD, PhD,^{a,b,c} Julien Mazières, MD, PhD,^d Emmanuel Chamorey, MD, PhD,^e Simon Heeke, PhD,^{a,b,c,f} Jonathan Benzaquen, MD, PhD,^{c,g} Brice Thamphya, MSc,^e Jacques Boutros, MD,^g Angélica Tiotiu, MD, PhD,^{h,i} Julien Fayada, MSc,^b Jacques Cadranel, MD, PhD,^j Michel Poudenx, MD,^{g,k} Denis Moro-Sibilot, MD, PhD,^l Fabrice Barlesi, MD, PhD,^{m,n} Juliette Thariat, MD, PhD,^o Christelle Clément-Duchêne, MD, PhD,^p Pascale Tomasini, MD, PhD,^m Véronique Hofman, MD, PhD,^{a,b,c} Charles-Hugo Marquette, MD, PhD,^{c,g} Paul Hofman, MD, PhD,^{a,b,c,*} On behalf of the STALKLUNG01 Study Consortium Investigators

^aLaboratory of Clinical and Experimental Pathology, FHU OncoAge, Centre Hospitalier Universitaire de Nice, Université Côte d'Azur, Nice, France

*Corresponding author.

Disclosure: Dr. Ilié reports receiving speaker's bureau honoraria from Roche, Merck & Co., AstraZeneca, Bristol-Myers Squibb, and Boehringer Ingelheim outside of the submitted work. Dr. Mazières reports receiving commercial research grants from AstraZeneca and Roche outside of the submitted work. Dr. Cadranel reports receiving commercial research grants and speaker's bureau honoraria from AstraZeneca, Bristol-Myers Squibb, Eli Lilly & Co., Novartis, Merck Sharp & Dohme, Roche, Takeda, Boehringer Ingelheim, and Pfizer outside of the submitted work. Dr. Poudenx reports receiving speaker's bureau honoraria from Roche, Bristol-Myers Squibb, Merck Sharp & Dohme, and Pfizer and has been an investigator for Novartis and Merck Sharp & Dohme outside of the submitted work. Dr. Moro-Sibilot reports receiving commercial research grants and honoraria from Roche, Bristol-Myers Squibb, Merck Sharp & Dohme, and Eli Lilly & Co. outside of the submitted work. Dr. Barlesi reports receiving speaker's bureau honoraria from AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Clovis Oncology, Eli Lilly Oncology, Hoffman-Roche, Novartis, Merck, Merck Sharp & Dohme, Pierre Fabre, and Pfizer outside of the submitted work. Dr. Tomasini reports receiving grants and nonfinancial support from Roche, AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, and Takeda outside of the submitted work. Dr. Heeke has received honoraria from Qiagen and Boehringer Ingelheim outside of the submitted work. Dr. Hofman has received honoraria from Roche, AstraZeneca, Bristol-Myers Squibb, Pfizer, Novartis, Bayer, Merck, Merck Sharp & Dohme, Qiagen, Illumina, Thermo Fisher Scientific, and Biocartis outside of the submitted work. The remaining authors declare no conflict of interest.

On behalf of the STALKLUNG01 Study Consortium Investigators: Dominique Israel-Biet, MD, PhD (Hôpital Européen Georges Pompidou), Christophe Pison, MD, PhD, Denis Moro-Sibilot, MD, PhD, Sylvie Lantuejoul, MD, PhD, Olivier Stephanov, MD, PhD, Meyzenc Juliette, Christophe Mendozat, Manel Zaidi, Sandra Coulouvrat, Edwige Col (Hôpital Universitaire de Grenoble), Pascal Chanez, MD, Laurent Greillier, MD, PhD, Pascale Tomasini, MD, PhD, Fabrice Barlesi, MD, PhD, Céline Mascaux, MD, PhD, Sandrine Jourdan, Aurélie Roger, Julie Biemar, Rondo Randriamampionona (APHM Hôpitaux Universitaires de Marseille), François Chabot, MD, Angélica Tiotiu, MD, Christelle Clément-Duchêne, MD, Jean-Michel Vignaud, Stéphanie Lacomme, Sandra Lomazzi (Hôpital Universitaire de Nancy), Carine Laurent, Xavier Bulsei, Laura Bischoff, Raymond Rakotonirina, Mehdi Layouni (Institut de Cancérologie de Lorraine) Gaëtan Deslee, MD (Hôpital Universitaire de Reims), Hervé Mal, MD (Hôpital Bichat), Romain Kessler, MD (Hôpital Universitaire de

Strasbourg), Jean-Michel Vergnon, MD, Isabelle Pelissier, MD (Hôpital Universitaire de St Etienne), Antoine Cuvelier, MD (Hôpital Universitaire de Rouen), Arnaud Bourdin, MD (Hôpital Universitaire de Montpellier), Vincent Jounieaux, MD (Hôpital Universitaire d'Amiens), Nicolas Roche, MD (Hôpital Cochin), Stéphane Jouneau, MD (Hôpital Universitaire de Rennes), Philippe Bonniaud, MD (Hôpital Universitaire de Dijon), Arnaud Scherpereel, MD, PhD (Hôpital Universitaire de Lille), Jean François Mornex, MD, PhD (Hôpital Universitaire de Lyon), François Steenhoutwer, MD (Hôpital de Roubaix), Sylvie Leroy, MD, Charles Hugo Marquette, MD, PhD, Jonathan Benzaquen, MD, PhD, Andrea Mazzette, MD, Bernard Padovani, MD, PhD, Paul Hofman, MD, PhD, Marius Ilié, MD, PhD, Véronique Hofman, MD, PhD, Julien Fayada, Elodie Long-Mira, MD, PhD, Sandra Lassalle, MD, PhD, Johanna Pradelli, MD, Estelle Martinez, Marine Habault, Mélanie Bonnard, Julie Moutarde, Rachida Yatimi, Hakima Labsi, Loïc Gazoppi, Tumorothèque Lpce, Jennifer Griffonnet, Maureen Fontaine, Ariane Guillemart, Catherine Butori, MD, Eric Selva, (Centre Hospitalier Universitaire de Nice), Michel Poudenx, MD, Josiane Otto, MD, Christophe Hebert, MD, Delphine Botchiellini, MD, Soukaina Boudouf, Margaux Menier, Estelle Ocelli, Sophie Bellentani, Carine Pion, Elodie Fournier (Centre Antoine Lacassagne de Nice), Juliette Thariat, MD, PhD, Radj Gervais, Karim Hamond (Centre François BACLESSE à Caen), Sylvain Marchand-Adam, MD, Laurent Plantier, Gaëlle Fajolle, Mélanie Rayez (Hôpital Universitaire de Tours), Jacques Cadranel, MD, PhD, Vincent Fallet, MD, Marie Wislez, MD, PhD, Martine Antoine, Jean-François Cote, Nouha Chaabane, MD, Anne Marie Ruppert, MD, Eliane Bertrand, Anita Rodenas, Gwenaëlle Pontdeme, Nathalie Mathiot, Tamazouzt Ribert (Hôpital Tenon), Julien Mazières, MD, PhD, Nicolas Guibert, MD, PhD, Damien Rouviere, MD, Emilie Bousquet, MD, Laurence Bigay-Game, MD, Christophe Hermant, MD, Gavin Plat, MD, Isabelle Rouquette, MD, Solène Evrard, MD, Sandrine Gouin, Estelle Taranchon Clermont, Inge Dormoy, Christelle Coulomb, Anne Pradine, Véronique Lambert (Hôpital Universitaire de Toulouse), Lilian Laborde (Institut Paoli-Calmettes de Marseille), Olivier Castelnaud, MD (Institut Tzanck de St Laurent du Var)

Address for correspondence: Paul Hofman, MD, PhD, Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, 30 Avenue de la Voie Romaine, BP69, 06001 Nice cedex 01, France. E-mail: hofman.p@chu-nice.fr

© 2021 International Association for the Study of Lung Cancer. Published by Elsevier Inc. All rights reserved.

ISSN: 1556-0864

<https://doi.org/10.1016/j.jtho.2021.01.1617>

^bHospital-Related Biobank (BB-0033-00025), FHU OncoAge, Centre Hospitalier Universitaire de Nice, Université Côte d'Azur, Nice, France

^cInstitute of Research on Cancer and Ageing of Nice (IRCAN), CNRS, INSERM, FHU OncoAge, Université Côte d'Azur, Nice, France

^dDepartment of Pulmonology, Centre Hospitalier Universitaire Toulouse, Institut Universitaire du Cancer, Université Paul Sabatier, Toulouse, France

^eBiostatistics Unit, Antoine Lacassagne Comprehensive Cancer Center, Nice, France

^fDepartment of Thoracic/Head & Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas

^gDepartment of Pulmonary Medicine and Oncology, FHU OncoAge, Centre Hospitalier Universitaire de Nice, Université Côte d'Azur, Nice, France

^hDepartment of Pulmonology, Centre Hospitalier Régional Universitaire de Nancy, Nancy, France

ⁱDevelopment, Adaptation and Disadvantage, Cardio-Respiratory Regulations and Motor Control, Université de Lorraine, Nancy, France

^jDepartment of Pulmonology, APHP, Hôpital Tenon and GRC04 Therascan, Sorbonne Université, Paris, France

^kDepartment of Oncology, Centre Antoine Lacassagne, Nice, France

^lThoracic Oncology Unit, Centre hospitalier universitaire Grenoble-Alpes, Grenoble, France

^mCentre d'Essais Précoces en Cancérologie de Marseille CLIP², Aix Marseille University, CNRS, INSERM, CRCM, APHM, Marseille, France

ⁿDepartment of Cancer Medicine, Institut Gustave Roussy, Villejuif, France

^oDepartment of Radiation Therapy, Centre François Baclesse-ARCHADE, Université de Caen Normandie, Caen, France

^pOncology Department, Institut de Cancérologie de Lorraine, Nancy, France

Received 30 September 2020; revised 18 January 2021; accepted 22 January 2021

Available online - 3 February 2021

ABSTRACT

Introduction: Patients with advanced-stage NSCLC whose tumors harbor an *ALK* gene rearrangement benefit from treatment with multiple *ALK* inhibitors (ALKi). Approximately 30% of tumor biopsy samples contain insufficient tissue for successful *ALK* molecular characterization. This study evaluated the added value of analyzing circulating tumor cells (CTCs) as a surrogate to *ALK* tissue analysis and as a function of the response to ALKi.

Methods: We conducted a multicenter, prospective observational study (NCT02372448) of 203 patients with stage IIIB/IV NSCLC across nine French centers, of whom 81 were *ALK* positive (immunohistochemistry or fluorescence in situ hybridization [FISH]) and 122 *ALK* negative on paraffin-embedded tissue specimens. Blood samples were collected at baseline and at 6 and 12 weeks after ALKi initiation or at disease progression. *ALK* gene rearrangement was evaluated with CTCs using immunocytochemistry and FISH analysis after enrichment using a filtration method.

Results: At baseline, there was a high concordance between the detection of an *ALK* rearrangement in the tumor tissue and in CTCs as determined by immunocytochemistry (sensitivity, 94.4%; specificity 89.4%). The performance was lower for the FISH analysis (sensitivity, 35.6%; specificity, 56.9%). No significant association between the baseline levels or the dynamic change of CTCs and overall survival (hazard ratio = 0.59, 95% confidence interval: 0.24–1.5, $p = 0.244$) or progression-free survival (hazard ratio = 0.84, 95% confidence interval: 0.44–1.6, $p = 0.591$) was observed in the patients with *ALK*-positive NSCLC.

Conclusions: CTCs can be used as a complementary tool to a tissue biopsy for the detection of *ALK* rearrangements.

Longitudinal analyses of CTCs revealed promise for real-time patient monitoring and improved delivery of molecularly guided therapy in this population.

© 2021 International Association for the Study of Lung Cancer. Published by Elsevier Inc. All rights reserved.

Keywords: *ALK*; CTCs; Immunocytochemistry; FISH; Lung adenocarcinoma

Introduction

NSCLC harboring *ALK* receptor tyrosine kinase (*ALK*) rearrangements is a distinct subset of lung cancers, occurring in approximately 4% of patients at an advanced stage.¹ Several *ALK* inhibitors (ALKi) have been approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of patients with *ALK*-positive NSCLC at first or latter lines of treatment.^{2,3}

The eligibility of patients with NSCLC to receive ALKi relies on a companion immunohistochemistry (IHC) performed on tumor tissue,⁴ for example, IHC or fluorescence in situ hybridization (FISH) that needs a sufficient number of tumor cells to be conclusive. However, procedures providing small biopsy samples (e.g., fine-needle aspiration, transthoracic needle biopsy, endobronchial ultrasound, bronchoscopy, bronchial biopsies) may lack a sufficient number of tumor cells. Furthermore, invasive procedures can be related to a relative risk of morbidity. In addition, because tumors evolve and adapt to targeted therapies, it is becoming increasingly important to monitor the molecular changes occurring in

patients with NSCLC in real-time, especially when selecting subsequent lines of treatment.⁵ Moreover, the degree of benefit from ALKi varies widely between patients with NSCLC, despite sharing molecular drivers. Longitudinal biopsies on disease progression have been useful in identifying the molecular mechanisms driving resistance to ALKi. Yet, repeated tissue biopsies are not always feasible and sampling of a single tumor site may not reflect the spatial heterogeneity of resistance mechanisms.⁶

In daily practice, accurate identification of *ALK* fusions and tumor evolution during therapy could have significant impact on the therapeutic strategy, likely to have the greatest impact on patient outcome.⁶ Therefore, developing noninvasive surrogate methods suitable to detect *ALK* rearrangements at baseline and during tumor evolution can be pivotal to improving the success rate of ALKi.

It is now well admitted that liquid biopsy (LB) is a simple, noninvasive, and easily repeatable alternative to tumor biopsies to assess the tumor's molecular status and to monitor genetic changes in real time.⁷ Among the LB components, circulating tumor cells (CTCs) contain tumorigenic cell clones with high relevance for metastatic dissemination.⁸ CTCs are likely released from distinct metastatic sites and may inform on the genomic heterogeneous picture of the metastatic disease. In contrast to tumor biopsies, CTCs could allow longitudinal monitoring at different time points during treatment, thereby having the potential to guide therapeutic decisions on real time.⁹

We and other groups have reported the feasibility of *ALK* rearrangement detection with CTCs collected from patients with *ALK*-positive NSCLC at baseline by using a

filter-based technique for CTC enrichment and *ALK* testing by FISH or immunocytochemistry (ICC).¹⁰⁻¹² To confirm these previous retrospective feasibility studies, we performed the first multicenter, prospective clinical validation of the use of CTCs as a surrogate for tumor tissue for *ALK* testing, thus aiding in prognostication and therapy selection at baseline and during treatment with ALKi of patients with stage IIIB/IV NSCLC.

Materials and Methods

Patients and Samples

This multicenter, prospective observational study (NCT02372448) was conducted across nine French clinical institutions from January 23, 2015, to November 21, 2019, in accordance with the Declaration of Helsinki and the European Directive and Good Clinical Practices. The study was approved by the Ethics Committee (Comité de Protection des Personnes Sud-Méditerranée V) and authorized by the Agence nationale de sécurité du médicament et des produits de santé. Informed written consent was obtained from all the patients. Eligible patients had histologically confirmed stage IIIB/IV NSCLC (Table 1). A few number of patients had initially been diagnosed with having stages I to IIIA NSCLC, enrolled in the study at the time of the metastatic relapse (Table 1). For these patients, a rebiopsy was performed to evaluate the *ALK* status. These patients may have had previous chemotherapy delivered as part of their primary treatment, but they must have completed primary therapy at least 3 months before their entry in the study.

The prespecified primary outcome was sensitivity and specificity of FISH between tumor tissue and CTCs at baseline, follow-up (visit 1) after 6 weeks (V1), and follow-up (visit 2) after 12 weeks (V2). Prespecified secondary outcomes included sensitivity and specificity of ICC at baseline, V1, and V2 and association of *ALK*-positive CTCs and clinical outcome.

ALK rearrangement was locally tested on the tumor tissue by IHC (anti-*ALK* antibody, prediluted, clone D5F3, Roche Diagnostics, Basel, Switzerland) and FISH (Vysis *ALK* Break Apart Rearrangement Probe Kit; Abbott Molecular Inc., Abbott Park, IL), performed according to prespecified standard operating procedures in the International Organization for Standardization 15189-accredited laboratories (accreditation no. 8-3034; <https://tools.cofrac.fr/annexes/sect8/8-3034.pdf>).

A 30 mL of peripheral blood was collected in ethylenediaminetetraacetic acid or Streck tubes at baseline (V0) and at 6 weeks (V1) and 12 weeks (V2) on ALKi initiation or at disease progression.¹³ CTCs were enumerated on ISET filters (Isolation by Size of Tumor Cells, Rarecells, Paris, France), as previously reported.^{10,14} Briefly, the stained filters were examined by

Table 1. Main Clinicopathologic Data of the 203 Patients With NSCLC Included in This Study

Clinicopathologic Features	ALK Negative n (%)	ALK Positive n (%)
Overall	122 (60)	81 (40)
Age (median, range)	64 (33-92)	62 (29-82)
Sex		
Male	85 (70)	39 (48)
Female	37 (30)	42 (52)
Smoking status		
Current smoker	49 (40)	6 (7)
Former smoker	65 (53)	30 (37)
Never smoker	8 (7)	45 (56)
Histological subtype		
Adenocarcinoma	98 (80)	80 (99)
Squamous cell carcinoma	15 (12)	0 (0)
NSCLC NOS	9 (8)	1 (1)
Disease status at study inclusion		
Metastatic relapse ^a	1 (1)	9 (11)
Stage IIIB	17 (14)	10 (12)
Stage IV	104 (85)	62 (77)

NOS, not otherwise specified.

^aCases with initially diagnosed stage I to IIIA NSCLC with metastatic relapse and rebiopsy to evaluate the *ALK* status.

a senior thoracic cytopathologist using light microscopy in the following two steps: (1) screening at $\times 20$ magnification to perform cell localization and detection, and then, (2) observation at $\times 100$ magnification using an oil immersion for detailed cytomorphological analysis. CTCs were defined as cells presenting all the following criteria: (1) nuclear size equal or larger than one pore (i.e., equal or larger than $8\text{ }\mu\text{m}$ in diameter); (2) irregularity of the nuclear contour; (3) presence of a well-defined and visible cytoplasm; and (4) high nuclear-to-cytoplasmic ratio (>0.8).

Consequently, computed tomography scans or magnetic resonance imagings were performed at baseline and during follow-up. The response to ALKi (Supplementary Table 1) was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. The disease control rate was defined as a complete or partial response or stable disease on the basis of RECIST.

ALK ICC and FISH on ISET-Enriched CTCs

All ISET filters were sent to the coordinating team at the CHU Nice for centralized ALK analysis. ICC and FISH were performed on CTCs isolated using the ISET method on unstained spots of the corresponding filters containing CTCs detected on four spots colored with May-Grünwald-Giemsa.¹⁵ Three spots were used for ICC and three spots were used for FISH per filter. For ICC, the spots were incubated with a primary antibody against the ALK protein (prediluted, clone D5F3; Ventana) for 32 minutes at room temperature. FISH analysis was carried out using a break-apart probe for the ALK gene (Vysis ALK Break Apart Rearrangement Probe Kit) in accordance with the manufacturer's instructions. Cells revealing at least one split signal or single 3' signals

were considered positive for an ALK rearrangement. ISET filters were examined independently and blinded to clinical, IHC, ICC, and FISH data and tissue genotype. We used the human NSCLC cell line H2228 obtained from the American Type Culture Collection (Manassas, VA) as an ALK rearrangement-positive control in every run.¹⁰

Statistical Analysis

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

The association to clinical variables was assessed using chi-square test, Student's *t* test, and Fisher's exact test analyses.

Progression-free survival was defined as the time between the date of the start of ALKi and the date of clinical or radiologic progression according to RECIST or death. Overall survival was defined as the time between the date of the start of ALKi and the date of death. Patients who were progression free and alive at the time of analysis were censored.

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

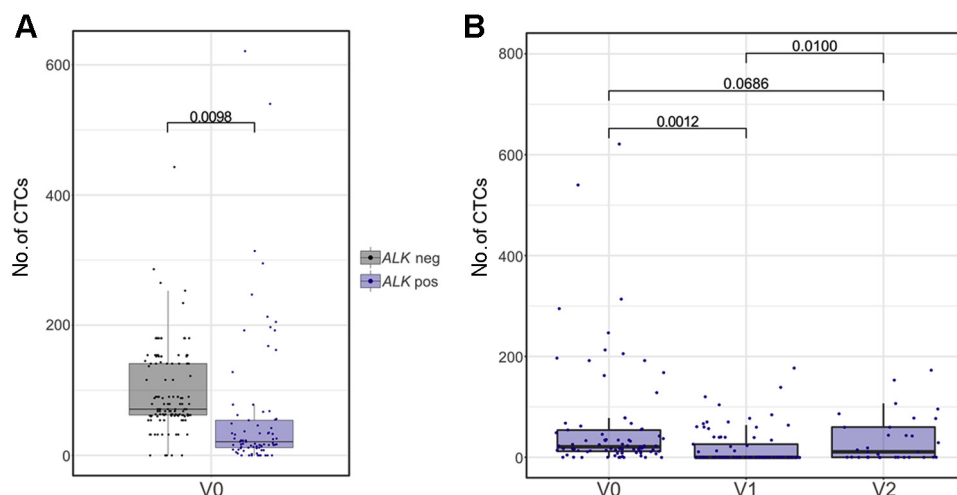


Figure 1. Boxplots revealing the levels of CTCs (A) according to the ALK status at baseline (visit 0 (V0) and (B) during the monitoring of patients with ALK-pos NSCLC at follow-up (visit 1) after 6 weeks (V1) and follow-up (visit 2) after 12 weeks (V2). CTC, circulating tumor cell; neg, negative; pos, positive.

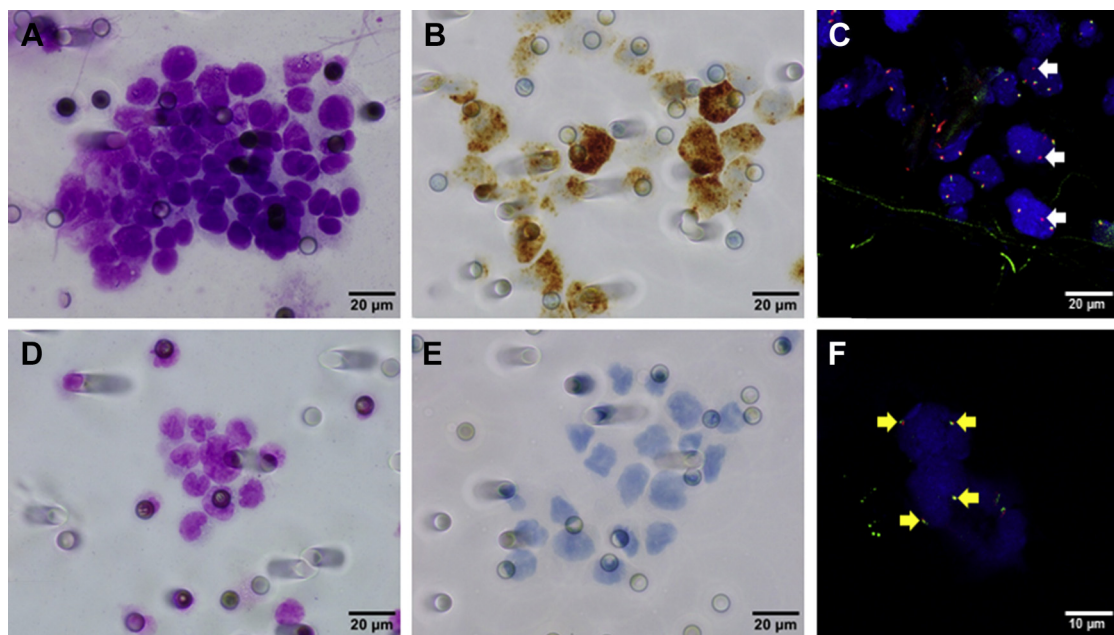


Figure 2. Representative images of (A-C) *ALK*-positive and (D-F) *ALK*-negative NSCLC cases. (A) CTCs revealing malignant cytomorphic criteria isolated by the ISET method (original magnification $\times 200$; MGG staining; bar: 20 μm). (B) CTCs revealing an intense and cytoplasmic staining (score 3+) (*ALK* immunostaining using D5F3 mAb, immunoperoxidase; original magnification $\times 200$; bar: 20 μm). (C) CTC nuclei hybridized with the Vysis *ALK* Break Apart Rearrangement Probe Kit. The two probes (3' red; 5' green) reveal a distinct separation of the red and green signals (arrows) indicating a rearrangement in the 2p23 *ALK* gene locus (original magnification $\times 1000$; bar: 20 μm). (D) Circulating cells revealing malignant cytomorphic criteria isolated by the ISET method (original magnification $\times 200$; MGG staining; bar: 20 μm). (E) CTCs revealing lack of staining (score 0) (D5F3 mAb, immunoperoxidase; original magnification $\times 200$; bar: 20 μm). (F) Circulating tumor cell nuclei hybridized with the Vysis *ALK* Break Apart Rearrangement Probe Kit. The probes give overlapping signals in the nuclei without the rearrangement (arrows; original magnification $\times 1000$; bar: 20 μm). CTC, circulating tumor cell; mAb, monoclonal antibody; MGG, May-Grünwald-Giemsa.

Results

Patient Characteristics

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

At baseline (V0), CTCs were detected in 187 patients (92%) (range: 2–621 per 4 mL; median, 62 CTCs per 4 mL) (Fig. 1A). A significantly higher number of CTCs were detected in the patients with *ALK*-negative NSCLC than in the patients with *ALK*-positive NSCLC (median CTCs = 71 versus 21; $p = 0.0098$) (Fig. 1A). CTCs were not detected in 16 of 203 patients (8%); 10 of 203 patients with *ALK*-positive NSCLC (5%) and 6 of 203 patients with *ALK*-negative NSCLC (3%).

At the first visit (V1) during the monitoring of patients with *ALK*-positive NSCLC, 27 of 81 patients (33%) had CTCs (range: 6–177 per 4 mL; median, six CTCs per 4 mL) (Fig. 1B). At the second follow-up visit (V2), we

detected CTCs (range, 11–173 per 4 mL; median, 19 CTCs per 4 mL) in 17 of 81 patients with *ALK*-positive NSCLC (21%) (Fig. 1B).

Performance of CTCs for Detection of ALK Rearrangements

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

Moreover, the levels of CTCs or *ALK*-positive CTCs significantly decreased with ALKi treatment (Fig. 1B and Supplementary Fig. 1).

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

Table 2. Correlation Analysis Between Patients With *ALK*-Positive NSCLC With Detectable CTCs and Patients With *ALK*-Positive NSCLC Without Detectable CTCs

Clinicopathologic Features	<i>ALK</i> Positive CTCs < Median n (%)	<i>ALK</i> Positive CTCs ≥ Median n (%)	<i>p</i> Value
Overall	63 (77.7)	18 (22.3)	
Age (mean, SD)	61.6 (13.3)	52.3 (12.2)	0.009
Sex			0.4305
Male	32 (50.8)	7 (38.9)	
Female	31 (49.2)	11 (61.1)	
Smoking status			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)	
Histology			1
Adenocarcinoma	61 (96.8)	18 (100)	
Squamous cell carcinoma	1 (1.6)	0 (0)	
NSCLC NOS	1 (1.6)	0 (0)	
Stage			0.02
I/II	2 (3.2)	4 (22.2)	
III/IV	61 (96.8)	14 (77.8)	

CTC, circulating tumor cell; NOS, not otherwise specified.

Association Between *ALK*-CTCs and Clinical Parameters or Outcome

An exploratory analysis revealed a significant association between the status of *ALK*-CTCs, younger age, and nonsmoking status. *ALK*-positive CTCs were present more often in younger patients (mean, 60 versus 64 y, $p = 0.038$) and nonsmokers ($p < 0.0001$). All the other clinical parameters were not significantly associated with *ALK*-rearranged CTC counts at baseline. In addition, in the patients with *ALK*-positive NSCLC, the levels of CTCs significantly correlated to age and stage (Table 2).

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

For the univariate analysis, the level of CTCs at baseline was assigned into low and high groups according to their respective median in the patients with *ALK*-positive NSCLC. We did not observe a significant association between baseline CTC levels and overall survival (hazard ratio = 0.59, 95% confidence interval: 0.24–1.5, $p = 0.244$, Fig. 3A) or progression-free survival (hazard ratio = 0.84, 95% confidence interval: 0.44–1.6, $p = 0.591$) (Fig. 3B).

In addition, we considered the dynamic change in the CTC counts under treatment as a possible parameter to evaluate. The patients were assigned into the following two groups: 13 patients presented stable or increased numbers of *ALK*-CTCs (median increase = 36, range: 2–290), whereas 37 patients presented a decrease in *ALK*-CTCs (median decrease = –7; range: –250 to –280). However, we did not observe a statistically significant association between the dynamic change in the CTC number on *ALK*i and response to treatment with a

univariate analysis ($p = 0.268$; Fig. 4A and B, Supplementary Fig. 2, and Supplementary Fig. 3).

Discussion

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

Currently, the eligibility of patients with NSCLC for *ALK*i relies on an assay performed on a tumor sample collected by biopsy.¹⁵ This invasive procedure is associated with a non-negligible risk of morbidity.¹⁶ The detection of an *ALK* rearrangement is currently performed on small biopsies or fine-needle aspirates and can be hindered by the limited quantity of tissue or the number of tumor cells. Tumor tissue is difficult to obtain from patients with advanced/metastatic lung cancer for whom surgery is rarely a component of the treatment care. Thus, a subset of patients with NSCLC can be deprived of potentially more efficient therapies. Finding alternative and more effective means of diagnosing and monitoring *ALK* rearrangements is a critical issue to identify the patients who may benefit from treatment with *ALK*i. For instance, detection of *ALK* fusions in circulating tumor DNA (ctDNA) has been previously tested using next-generation sequencing (NGS), reverse transcriptase polymerase chain reaction, or RNA sequencing with variable sensitivities (70%–80%) for

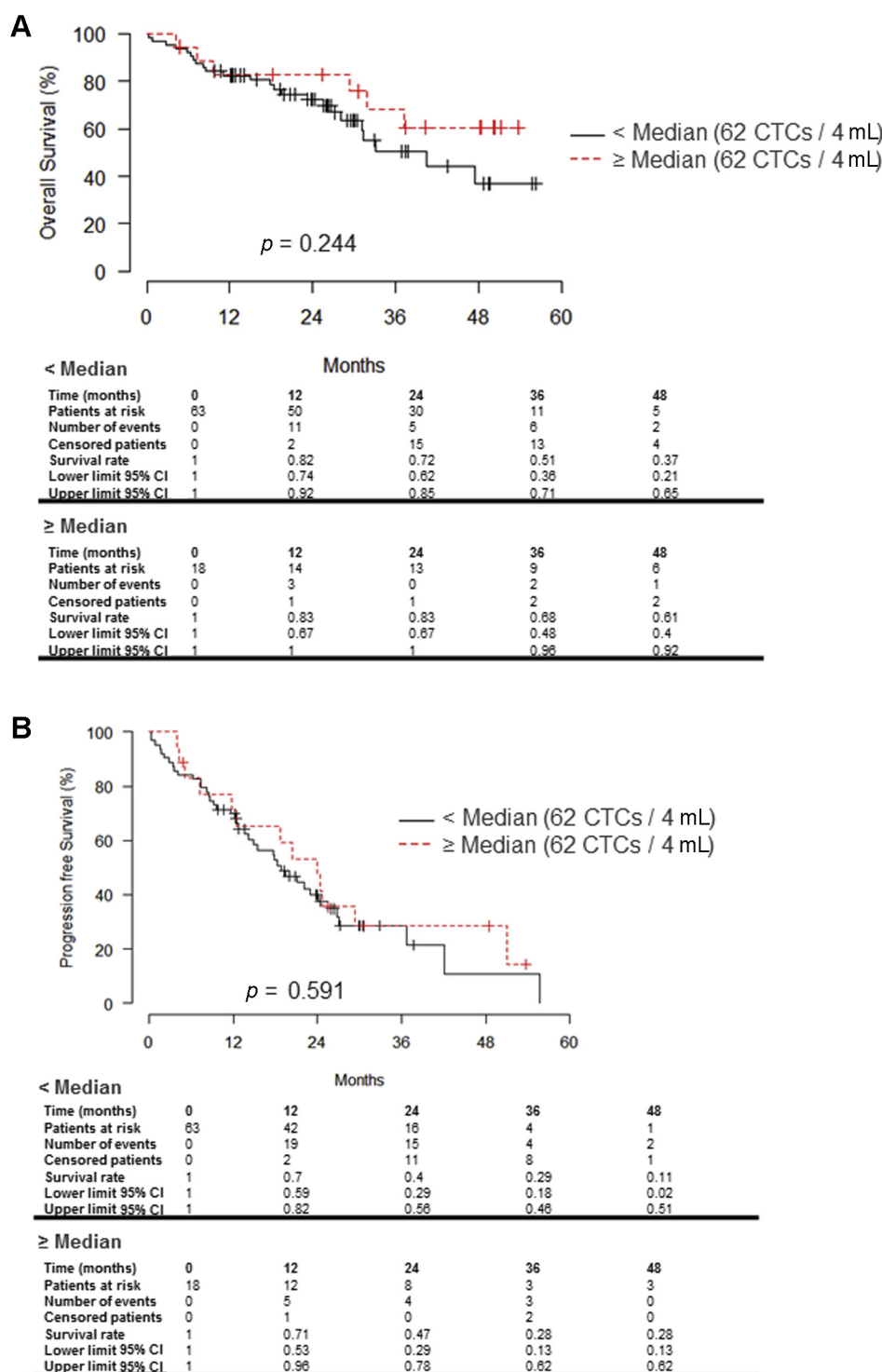


Figure 3. Kaplan-Meier survival curves according to the median of CTC level ($n = 62$ CTCs per 4 mL) at baseline from the 81 patients with ALK-positive NSCLC. (A) Overall survival and (B) progression-free survival curves. The p values were calculated using the log-rank test. CI, confidence interval; CTC, circulating tumor cell.

the different assays.^{17,18} Thus, in contrast to *EGFR* mutations, detection of *ALK* rearrangements with ctDNA is rarely implemented in daily practice.

Recently, the prospective, multicenter NILE study revealed that ctDNA can rescue biomarker-positive

patients with nondiagnostic tissue results, suggesting the clinical use of ctDNA in newly diagnosed metastatic NSCLC.¹⁹ However, the LB test used was compared with a standard-of-care tissue genotyping test and not the tissue-based NGS, and the results were only applicable to

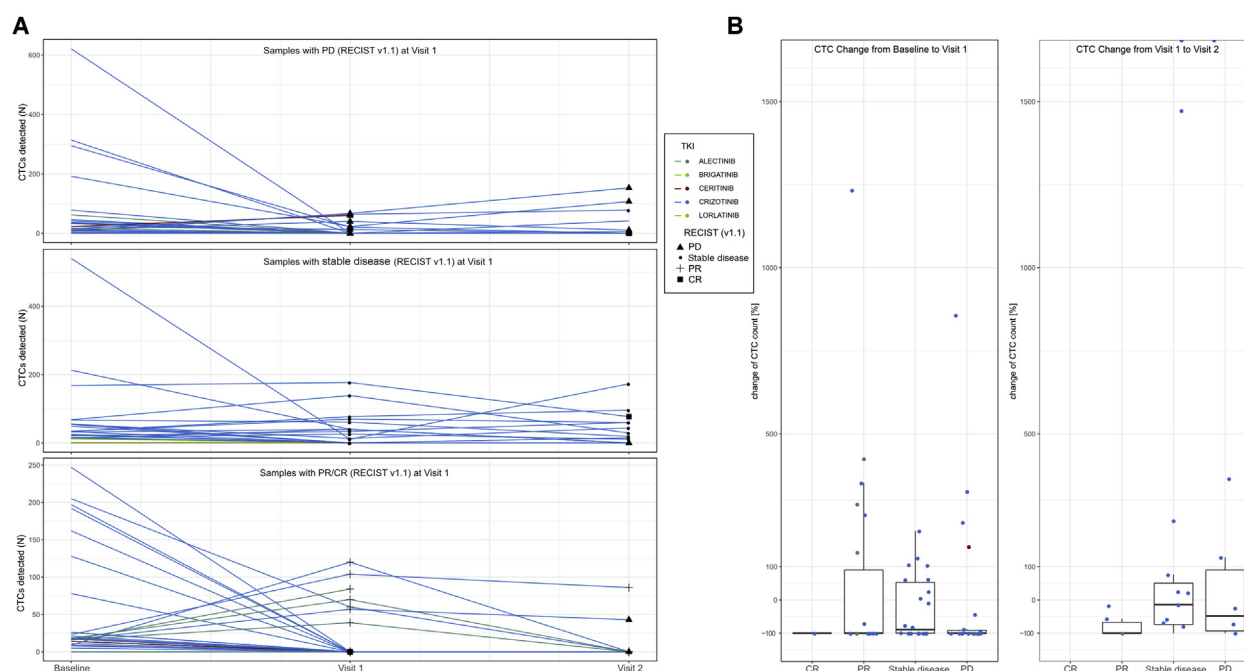


Figure 4. Plots of ALK-CTC counts per 3 mL of blood on the basis of ICC detection in patients who were serially sampled (e.g., paired time points for the same patient) during treatment with ALK inhibitors. (A) Spaghetti plots depicting the ALK-CTC counts in relation to PD, stable disease, and PR/CR on separate panels. (B) Boxplots depicting ALK-CTC changes in relation to CR, PR, stable disease, PD from baseline to visit 1, and from visit 1 to visit 2. The color of the lines is highlighting the respective TKI used at baseline for the treatment of each patient. The dots highlight the response according to RECIST v1.1 at the two respective follow-up visits (visit 1 and visit 2). Only patients with an ALK-positive tumor are included in the analysis. CR, complete response; CTC, circulating tumor cell; ICC, immunocytochemistry; N, number; PD, progressive disease; PR, partial response; RECIST v1.1, Response Evaluation Criteria in Solid Tumors version 1.1; TKI, tyrosine kinase inhibitor.

the Guardant360 test (Guardant Health, Inc., Redwood City, CA) and not to other LB tests.¹⁹ In addition, the FoundationOneLiquid CDx assay (Roche Foundation Medicine, Cambridge, MA), a pan-cancer ctDNA-based comprehensive genomic profiling assay, was recently approved by the Food and Drug Administration.²⁰ However, the number of cases with an *ALK* rearrangement used for the analytic validation was quite low.²⁰

A LB of CTCs present in the blood and the characterization of their *ALK* status are appealing alternatives that meet the urgent needs of these patients. In addition, no CTC-based molecular test is currently available in routine clinical practice.²¹

In this setting, CTCs which have been found to be detectable by ISET in 80% to 100% of patients with late stages of lung cancer represent a noninvasive and easily accessible source of tumor material for assessing *ALK* rearrangement in a dynamic manner.^{14,22} In our study, we did not isolate CTCs in only 8% of patients, which is in the range of sensitivity described for the detection of CTCs by ISET approach in patients with NSCLC.^{14,22–24} The lack of CTC detection in these patients could be related to either technical or biological hurdles. It may be that patients truly present negative

for CTCs or CTCs may be lost at different times during the filtration and staining process owing to (1) the pores of 8 μ m during the procedure of filtration or (2) the sequential washes used during the staining procedure performed after filtration.^{23,25} The high sensitivity and specificity of ISET were two essential starting points for the feasibility of the present independent and multicenter project.¹⁰ In our first feasibility monocentric study, we found excellent concordance for the *ALK* status between CTCs and the matched tumor tissue sample in a limited series of 87 patients with advanced NSCLC, with only five patients harboring an *ALK* rearrangement.¹⁰

In this multicenter, prospective observational study across nine centers, there was perfect concordance (100%) between the tissue and CTC *ALK* analyses, in all cases with CTCs isolated on filters (92%). In the small proportion of cases without CTCs, the *ALK* status could not be determined on the filters, suggesting technical challenges rather than biological heterogeneity, as described previously.²³

Our study presents some limitations. One ISET spot contains on average of 5000 white blood cells, mainly leukocytes, and only a small number of CTCs.²⁶

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

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

It is worth noting that other noninvasive biological components that can be isolated from peripheral blood, such as ctDNA, circulating exosomes, platelet RNA, or circulating tumor RNA, are currently being investigated for the detection of gene rearrangements,^{18,27,28} although the sensitivity and precision of these strategies need to be further evaluated and compared with those of CTC assays.

Although each of these approaches has the potential to provide novel diagnostic information and their exploration is highly encouraged, ctDNA certainly represents the most mature example of the investigation of LB in clinical practice for patients with NSCLC, in particular for gene mutation detection.^{5,6,29,30}

However, the analysis of ctDNA requires extensive deep sequencing of genomic DNA for reliable detection of the chromosomal break-point.³¹ Moreover, digital droplet polymerase chain reaction, BEAMing, and NGS have been reported to have a promising sensitivity for the detection of *ALK* gene fusions, but they are more variable than those observed for point mutations and indels.³² Further studies should focus on orthogonal comparisons using CTCs, ctDNA, and circulating tumor RNA to better characterize the presence of *ALK* rearrangements and to validate the clinical use of detecting these genomic variants in plasma, notably in naïve-treated patients with no tumor tissue accessibility.

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

Acknowledgments

This study was financially supported by a governmental grant from the National Institute of Cancer (INCa PHRC;

STALKLUNG01 13-APN-01), the Cancéropôle PACA, and the Conseil Départemental des Alpes-Maritimes.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at <https://doi.org/10.1016/j.jtho.2021.01.1617>.

References

1. Shaw AT, Engelman JA. ALK in lung cancer: past, present, and future. *J Clin Oncol*. 2013;31:1105-1111.
2. Solomon BJ, Kim DW, Wu YL, et al. Final overall survival analysis from a study comparing first-line crizotinib versus chemotherapy in ALK-mutation-positive non-small-cell lung cancer. *J Clin Oncol*. 2018;36:2251-2258.
3. Peters S, Camidge DR, Shaw AT, et al. Alectinib versus crizotinib in untreated ALK-positive non-small-cell lung cancer. *N Engl J Med*. 2017;377:829-838.
4. Planchard D, Popat S, Kerr K, et al. Metastatic non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2018;29(suppl 4):iv192-iv237.
5. Horn L, Whisenant JG, Wakelee H, et al. Monitoring therapeutic response and resistance: analysis of circulating tumor DNA in patients with ALK+ lung cancer. *J Thorac Oncol*. 2019;14:1901-1911.
6. Dagogo-Jack I, Brannon AR, Ferris LA, et al. Tracking the evolution of resistance to ALK tyrosine kinase inhibitors through longitudinal analysis of circulating tumor DNA. *JCO Precis Oncol*. 2018;2018:1-14.
7. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet*. 2019;20:71-88.
8. Krebs MG, Metcalf RL, Carter L, Brady G, Blackhall FH, Dive C. Molecular analysis of circulating tumour cells-biology and biomarkers. *Nat Rev Clin Oncol*. 2014;11:129-144.
9. Pantel K, Alix-Panabières C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. *Nat Rev Clin Oncol*. 2019;16:409-424.
10. Ilie M, Long E, Butori C, et al. ALK-gene rearrangement: a comparative analysis on circulating tumour cells and tumour tissue from patients with lung adenocarcinoma. *Ann Oncol*. 2012;23:2907-2913.
11. Paillet E, Adam J, Barthélémy A, et al. Detection of circulating tumor cells harboring a unique ALK rearrangement in ALK-positive non-small-cell lung cancer. *J Clin Oncol*. 2013;31:2273-2281.
12. Tan CL, Lim TH, Lim T, et al. Concordance of anaplastic lymphoma kinase (ALK) gene rearrangements between circulating tumor cells and tumor in non-small cell lung cancer. *Oncotarget*. 2016;7:23251-23262.
13. Ilie M, Hofman V, Leroy S, et al. Use of circulating tumor cells in prospective clinical trials for NSCLC patients - standardization of the pre-analytical conditions. *Clin Chem Lab Med*. 2018;56:980-989.

14. Hofman V, Bonnetaud C, Ilié MI, et al. Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker. *Clin Cancer Res*. 2011;17:827-835.
15. Shaw AT, Yeap BY, Mino-Kenudson M, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol*. 2009;27:4247-4253.
16. Huang MD, Weng HH, Hsu SL, et al. Accuracy and complications of CT-guided pulmonary core biopsy in small nodules: a single-center experience. *Cancer Imaging*. 2019;19:51.
17. Wang Y, Tian PW, Wang WY, et al. Noninvasive genotyping and monitoring of anaplastic lymphoma kinase (ALK) rearranged non-small cell lung cancer by capture-based next-generation sequencing. *Oncotarget*. 2016;7:65208-65217.
18. Park CK, Kim JE, Kim MS, et al. Feasibility of liquid biopsy using plasma and platelets for detection of anaplastic lymphoma kinase rearrangements in non-small cell lung cancer. *J Cancer Res Clin Oncol*. 2019;145:2071-2082.
19. Leighl NB, Page RD, Raymond VM, et al. Clinical utility of comprehensive cell-free DNA analysis to identify genomic biomarkers in patients with newly diagnosed metastatic non-small cell lung cancer. *Clin Cancer Res*. 2019;25:4691-4700.
20. Woodhouse R, Li M, Hughes J, et al. Clinical and analytical validation of FoundationOne Liquid CDx, a novel 324-Gene cfDNA-based comprehensive genomic profiling assay for cancers of solid tumor origin. *PLoS One*. 2020;15:e0237802.
21. Alix-Panabieres C. The future of liquid biopsy. *Nature*. 2020;579:S9.
22. Hofman P. ALK status assessment with liquid biopsies of lung cancer patients. *Cancers (Basel)*. 2017;9:106.
23. Farace F, Massard C, Vimond N, et al. A direct comparison of CellSearch and ISET for circulating tumour-cell detection in patients with metastatic carcinomas. *Br J Cancer*. 2011;105:847-853.
24. Krebs MG, Hou JM, Sloane R, et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. *J Thorac Oncol*. 2012;7:306-315.
25. Krebs MG, Sloane R, Priest L, et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. *J Clin Oncol*. 2011;29:1556-1563.
26. Chami F, Rothwell DG, McGranahan N, et al. Pulmonary venous circulating tumor cell dissemination before tumor resection and disease relapse [published correction appears in *Nat Med*. 2020;26:1147]. *Nat Med*. 2019;25:1534-1539.
27. Rolfo C, Mack PC, Scagliotti GV, et al. Liquid biopsy for advanced non-small cell lung cancer (NSCLC): a statement paper from the IASLC. *J Thorac Oncol*. 2018;13:1248-1268.
28. Hofman V, Heeke S, Marquette CH, Ilié M, Hofman P. Circulating tumor cell detection in lung cancer: but to what end? *Cancers (Basel)*. 2019;11:262.
29. Aggarwal C, Thompson JC, Black TA, et al. Clinical implications of plasma-based genotyping with the delivery of personalized therapy in metastatic non-small cell lung cancer. *JAMA Oncol*. 2019;5:173-180.
30. Zugazagoitia J, Ramos I, Trigo JM, et al. Clinical utility of plasma-based digital next-generation sequencing in patients with advance-stage lung adenocarcinomas with insufficient tumor samples for tissue genotyping. *Ann Oncol*. 2019;30:290-296.
31. McCoach CE, Blakely CM, Banks KC, et al. Clinical utility of cell-free DNA for the detection of ALK fusions and genomic mechanisms of ALK inhibitor resistance in non-small cell lung cancer. *Clin Cancer Res*. 2018;24:2758-2770.
32. Supplee JG, Milan MSD, Lim LP, et al. Sensitivity of next-generation sequencing assays detecting oncogenic fusions in plasma cell-free DNA. *Lung Cancer*. 2019;134:96-99.