STUDY PROTOCOL

General information	
Full study title and acronym	**Characterization of targeted therapy resistance mechanisms in EGFR/ALK/ROS1/BRAF-positive NSCLC by gene panel NGS in circulating cell-free DNA** V1.1, dated February 6th 2020
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Study duration	Study start (first subject first visit) is anticipated to take place in January 2020. Study completion is anticipated to take place in December 2020.
Study sites	Next generation sequencing of cfDNA samples will be performed at the Department of Genetics of IPO-Porto. Patients will be recruited from all the major hospitals and cancer institutes in Portugal.
1. Introduction, study rationale and study objective(s)	
Introduction and rationale	Lung cancer is the most common cause of cancer-related death among all human cancers, with two million new cases diagnosed worldwide in 2018 (Ferley et al, 2019). Non–small-cell lung cancer (NSCLC) accounts for more than 85% of lung cancers and the most common histology in this group is adenocarcinoma (Siegel et al, 2015). About 20% of adenocarcinomas have genomic changes in driver genes, namely, activating point mutations in EGFR or BRAF and gene fusions involving the ALK or ROS1 genes, which are predictive biomarkers for biological therapies having as targets the abnormal proteins coded by those somatic genetic changes (Hanna et al, 2017). Although such targeted therapies helped to significantly improve the prognosis of these molecular subtypes of NSCLC, multiple resistance mechanisms have been described after first-line targeted treatment, which may be highly relevant for the informed choice of second-line therapy. In NSCLCs with EGFR-sensitizing mutations, the most frequent mechanism of resistance to first-line anti-EGFR therapy is the emergence of the T790M EGFR mutation, which in turn is predictive of response to osimertinib (Planchard et al, 2018). Progression of ALK-positive NSCLC under treatment with first-line anti-ALK therapy is often associated with secondary point mutations in ALK, which may predict which second-line anti-ALK therapy is likely to be more effective. However, contrary to what is the case for EGFR-positive NSCLC, identification of the mechanism of resistance is recommended but not mandatory in ALK-positive NSCLC patients (Lindeman et al, 2018). The expanding number of mechanisms of resistance to biological therapies and the availability of several lines of treatment in NSCLC patients is creating the necessity for real-time tumor genotyping, yet tissue biopsies are

	difficult to perform serially and often yield inadequate DNA for next-generation sequencing (NGS). For instance, the guidelines include the option to start to test for the T790M EGFR mutation first in circulating tumor DNA (ctDNA) and to rebiopsy only if that mutation is not detected in ctDNA (Planchard et al, 2018). ctDNA mutation testing with NGS potentially facilitates the study of genotypically heterogeneous tumors and allows identification of multiple resistance mechanisms in NSCLC patients (Chabon et al, 2016), but the clinical relevance of this strategy requires additional studies. For ctDNA testing in patients progressing on first-line anti-EGFR therapy, we currently use the CE-IVD cobas® EGFR Mutation Test v2 for the qualitative detection and identification of mutations in EGFR exons 18, 19, 20, and 21, including the T790M mutation. In this setting and with this method, we detect a EGFR mutation in ctDNA in 66.6% of the patients, which is in line with a large metaanalysis (Li et al, 2014) showing 65% sensitivity (95% confidence interval: 61-68%). In this pilot study, we intend to evaluate the feasibility of using the AVENIO ctDNA Expanded Kit, a NGS liquid biopsy tumor profiling assay for identifying genomic aberrations derived from solid tumors, in NSCLC patients progressing after first-line targeted treatment. This investigational test analyses 77 genes, including those currently in the NCCN Guidelines and emerging biomarkers relevant to clinical research.
Study objective(s)	The primary objective of this pilot study is to evaluate the overall sensitivity of the AVENIO ctDNA Expanded Kit to detect the primary molecular change in NSCLC patients progressing after first-line targeted treatment with an approved targeted therapy for those molecular subtypes of NSCLC. The secondary objectives are: • To describe the pattern of resistance mechanisms in patients with EGFR, BRAF, ALK, or ROS1-positive NSCLC who progressed after first-line treatment. • To evaluate the rate of potential access to other approved or off-label targeted therapies. • To evaluate the potential impact of this strategy to reduce the rate of rebiopsies in these patients, as compared with historical data from the year before.
2. Investigational material	
Investigational material and comparator product(s)	The investigational assay to be used in this study is the AVENIO ctDNA Expanded Kit on a NextSeq 550 sequencer and the Oncology Analysis Server equipment, AVENIO software 2.0 and a report will be issued using NAVIFY Mutation Profiler software. The comparator product in this study with be the routine use of the CE-IVD cobas® EGFR Mutation Test v2, which will be used in parallel in ctDNA from patients with EGFR-positive NSCLC in progression. We will use the 95% confidence interval values (61-68%; mean 65%) reported in the metaanalysis of Li et al (2014) as the cut-off points for assessing superiority (>68%), inferiority (<61%) or non-inferiority (61-68%).
3. Study population	
Recruitment, enrollment	100 patients will be recruited from all major cancer hospitals in Portugal over a planned recruitment period of 12 months.

period, and sample size	
Inclusion criteria	Adult patients with EGFR, BRAF, ALK, or ROS1-positive NSCLC who progressed after first-line treatment with an approved targeted therapy for those molecular subtypes of NSCLC who have not yet initiated second-line therapy.
Exclusion criteria	• Patients without EGFR, BRAF, ALK, or ROS1-positive NSCLC. • Patients with EGFR, BRAF, ALK, or ROS1-positive NSCLC still responding to first-line treatment. • Patients with EGFR, BRAF, ALK, or ROS1-positive NSCLC who progressed after first-line treatment and have initiated second-line therapy. • Patients under the age of 18.
4. Study design and study procedure	
Study design	This will be an exploratory, multicenter, national, prospective, observational study.
Study procedure	Patients will be enrolled at the time of the Medical Oncology appointment in which progression after first-line targeted treatment is established. ctDNA testing for patients with EGFR-positive NSCLC will be ordered as usual and the surplus sample will be used for NGS. In the institutions where ctDNA EGFR testing is not routinely ordered and in patients with BRAF, ALK, or ROS1-positive NSCLC, ctDNA testing by NGS will be requested at the time of the first peripheral blood sample taken as part of his/her regular follow up. Peripheral blood samples for ctDNA testing will be collected in specific tubes for that purpose (to preserve ctDNA quality and avoid contamination with cellular DNA) and will be sent to the Department of Genetics of IPO-Porto as soon as possible (at room temperature). Samples from IPO Porto will be processed within 4 hours and samples from other hospitals will be sent by express mail and processed within 48 hours. Clinical decisions in patients with EGFR-positive NSCLC who progressed after first-line targeted treatment with an approved targeted therapy will be based on the ctDNA testing with the CE-IVD cobas® EGFR Mutation Test v2 whenever that test is already routinely used in any particular participating institution. The investigational assay AVENIO ctDNA Expanded Kit will be used in parallel in the surplus ctDNA sample and will be validated for its performance to detect EGFR mutations as compared with the current gold standard. In light of the potential higher sensitivity of the NGS test (limit of detection ≤1%) compared to the routine testing (limit of detection ≤5%) for EGFR mutations, this information will be provided to the treating clinician. Furthermore, whenever ctDNA testing is currently not routinely requested in EGFR, BRAF, ALK, or ROS1-positive NSCLC patients progressing after first-line treatment, the research information will be provided to the treating clinician. Given the recommended sample input for each test, two peripheral blood sample draws need to be collected for EGFR-positive NSCLC patients to ensure plasma input volume is sufficient for both cobas® EGFR Mutation Test v2 (2 ml) and AVENIO ctDNA Expanded Kit (4 ml).

	Subjects have the right to withdraw from the study at any time for any reason. If lost to follow-up, the assigned study staff will try to contact the subject by telephone followed by registered mail to establish and document as completely as possible the reason for the withdrawal. Subjects will be informed of circumstances under which their participation may be terminated by the responsible investigator without the subject's consent. The investigator may withdraw subjects from the study in the event of intercurrent illness, adverse events, lack of compliance with the study and/or study procedures (e.g., study visits), or any reason where it is felt that it is in the best interest of the subject to be terminated from the study. Any administrative or other reasons for withdrawal will be documented and explained to the subject. If the reason for removal of a subject from the study is an Adverse Event, the principal specific event will be recorded in the medical record.
5. Statistics	
Primary and secondary endpoint(s)	To evaluate the overall sensitivity of the AVENIO ctDNA Expanded Kit to detect the primary molecular change in NSCLC patients progressing after first-line targeted treatment with an approved targeted therapy for those molecular subtypes of NSCLC. The secondary endpoints are: • Number of resistance mechanisms in patients with EGFR, BRAF, ALK, or ROS1-positive NSCLC who progressed after first-line treatment. • The rate of potential access to other approved or off-label targeted therapies. • The rate of rebiopsies in these patients, as compared with historical data from the year before.
Statistical methods	Not applicable as it is a pilot study.
Sample size, level of significance, and power	Not applicable as it is a pilot study. We will use the 95% confidence interval values (61-68%; mean 65%) reported in the metaanalysis of Li et al (2014) as the cut-off points for assessing superiority (>68%), inferiority (<61%) or non-inferiority (61-68%). If statistical power falls short after first 100 patients, cohort could be extended.
6. Safety assessment	
	Not applicable as it is an observational study. Treatment is not part of the protocol but done at physicians' decision, so safety assessment is not applicable.
7. Compliance Statements	
	General requirements: The study will be conducted in compliance to this study protocol, the current version of the Declaration of Helsinki, ICH GCP, and applicable local legal and regulatory requirements. Submission of study documents: Before study start, the study protocol, and subject information / informed consent and any other study-related document as required by applicable laws and regulations will be submitted to the Ethics Committee and regulatory authorities for written approval.

	Any protocol amendments or new or amended information that requires ethical consideration will be submitted for written approval, too. In addition, a study report (interim and /or full report) will be submitted to regulatory authorities in line with applicable timelines. Subject information and informed consent: Subjects/legal representatives will be informed orally and in writing about the objectives of the study, study procedures, potential risks, and about the fact that to some extent data will be accessible for third parties (see below) for the purpose of controlling the study conduct - provided that data confidentiality is ensured at any time. Before any study-related activities are initiated, the subjects / legal representatives will have to sign the written informed consent. The participation in the study is entirely voluntary. The subjects have the right to withdraw their willingness to participate in the study at any time without affecting their future medical care in any way. Clinical study results and publication: The results of the clinical study will be documented in a clinical study report and if possible, will be published (e.g. in a journal or presented in a scientific meeting).
8. Data confidentiality and protection	
Data confidentiality and data protection	Auditors, Ethics Committee, and the regulatory authorities will be granted direct access to the subject's medical records to the extent permitted by the applicable law and regulation for verification of clinical study procedures, and/or data control, ensuring subject data confidentiality. The subject's file and the source data will be archived in line with national and international legal requirements.The key that allows patient identification will be eliminated 5 years after the end of the study.
9. Quality controls and assurance	
Quality controls and quality assurance	The gold standard test is the CE-IVD cobas® EGFR Mutation Test v2, which will be used according to the manufacturer's instructions. The Department of Genetics is regularly enrolled in external quality assessment using this test. The investigational assay to be used in this study is the AVENIO ctDNA Expanded Kit on a NextSeq 550 sequencer and the Oncology Analysis Server equipment. The performance in the detection of EGFR mutations will be measured against the gold standard test currently used for medical decisions. All other mutations will be validated by other methods whenever possible (such as qPCR, dPCR).
10. References	
Literature references	• Chabon JJ, Simmons AD, Lovejoy AF, Esfahani MS, Newman AM, Haringsma HJ, Kurtz DM, Stehr H, Scherer F, Karlovich CA, Harding TC, Durkin KA, Otterson GA, Purcell WT, Camidge DR, Goldman JW, Sequist LV, Piotrowska Z, Wakelee HA, Neal JW, Alizadeh AA, Diehn M: **Circulating tumour DNA profiling reveals heterogeneity of egfr inhibitor resistance mechanisms in lung cancer patients**. *Nat Commun* 2016, 7:11815. • Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Pineros M, Znaor A, Bray F: **Estimating the global cancer incidence and mortality in 2018: Globocan sources and methods**. *International journal of cancer Journal international du cancer* 2019, 144(8):1941-1953. • Hanna N, Johnson D, Temin S, Masters G: **Systemic therapy for stage**

iv non-small-cell lung cancer: American society of clinical oncology clinical practice guideline update summary. *Journal of oncology practice / American Society of Clinical Oncology* 2017, 13(12):832-837.

- Li Z, Zhang Y, Bao W, Jiang C. **Insufficiency of peripheral blood as a substitute tissue for detecting EGFR mutations in lung cancer: a meta-analysis.** *Target Oncol.* 2014 Dec;9(4):381-8.
- Lindeman NI, Cagle PT, Aisner DL, Arcila ME, Beasley MB, Bernicker EH, Colasacco C, Dacic S, Hirsch FR, Kerr K, Kwiatkowski DJ, Ladanyi M, Nowak JA, Sholl L, Temple-Smolkin R, Solomon B, Souter LH, Thunnissen E, Tsao MS, Ventura CB, Wynes MW, Yatabe Y: **Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors: Guideline From the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology**. *Arch Pathol Lab Med.* 2018 Mar;142(3):321-346.
- Planchard D, Popat S, Kerr K, Novello S, Smit EF, Faivre-Finn C, Mok TS, Reck M, Van Schil PE, Hellmann MD, Peters S: **Metastatic non-small cell lung cancer: Esmo clinical practice guidelines for diagnosis, treatment and follow-up**. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2019, 30(5):863-870.
- Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2015**. *CA: a cancer journal for clinicians* 2015, 65(1):5-29.