PROTOCOL:

proSpecTive sAmpling in dRiver muTation pulmonary oncology patients on Tyrosine Kinase Inhibitors (START-TKI)

Writing committee:
C.M.J. Steendam, MD, Department of Pulmonology
Prof. J.G.J.V. Aerts, MD/PhD, Department of Pulmonology
Prof. A.C. Dingemans, MD/PhD, Department of Pulmonology
Prof. R.H.N. van Schaik, PhD, Department of Clinical Chemistry
H.J. Dubbink, PhD, Department of Pathology
Prof. A.H.J. Mathijssen, MD/PhD, Department of Medical Oncology
S. Bins, MD/PhD, Department of Medical Oncology
G.D.M. Veerman, MD, Department of Medical Oncology
S.L.W. Koolen, PharmD/PhD, Department of Medical Oncology
E. Oomen – de Hoop, PhD, Department of Medical Oncology

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Coordinating investigators/project leader	**Christi M.J. Steendam, MD** **Department of Pulmonology, Room Rg-424** **Erasmus MC Cancer Institute Rotterdam** **Dr. Molewaterplein 40** **3015 GD Rotterdam, the Netherlands** **Tel: +31 624710745** **Email: c.steendam@erasmusmc.nl**
Principal investigator(s) (in Dutch: hoofdonderzoeker/ uitvoerder)	**Erasmus MC:** **Prof. J.G.J.V. Aerts, MD/PhD** **Department of Pulmonology, Room Rg-436** **Erasmus MC Cancer Institute Rotterdam** **Dr. Molewaterplein 40** **3015 GD Rotterdam, the Netherlands** **Tel: +31 10 7043777** **Amphia Breda:** **C.M.J. Steendam, MD** **Department of Pulmonology** **Molengracht 21**

	4818 CK, the Netherlands Tel: +31 76 595 2328
Other investigators/writing committee	Prof. A.C. Dingemans, MD/PhD, Department of Pulmonology Prof. R.H.N. van Schaik, PhD, Department of Clinical Chemistry H.J. Dubbink, PhD, Department of Pathology Prof. A.H.J. Mathijssen, MD/PhD, Department of Medical Oncology M. Paats, MD/PhD, Department of Pulmonology S. Bins, MD/PhD, Department of Medical Oncology S.L.W. Koolen, PharmD/PhD, Department of Medical Oncology G.D.M. Veerman, MD, Department of Medical Oncology E. Oomen – de Hoop, PhD, Department of Medical Oncology Trial Department Pulmonology
Sponsor (in Dutch: verrichter/opdrachtgever)	Erasmus MC, Department of Pulmonology, Department of Clinical Chemistry, Department of Pathology, Department of Medical Oncology
Subsidising party	Not applicable
Independent expert (s)	L.M. van den Toorn, MD/PhD Department of Pulmonology Erasmus MC, Rotterdam

Laboratory sites	International Reference Center Pharmacogenetics, Department of Clinical Chemistry, Erasmus MC, Rotterdam Laboratory for Molecular Diagnostics, Department of Pathology, Erasmus MC, Rotterdam Laboratory of Translational Pharmacology, Erasmus MC Cancer Institute, Rotterdam
Pharmacy	Not applicable

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Sponsor or legal representative: **Head of Department:** Prof Dr. J.G.J.V. Aerts		11/2/21
Principal Investigator: Prof. Dr. J.G.J.V. Aerts		11/2/21

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)
AE	Adverse Event
ALK	Anaplastic lymphoma kinase
AR	Adverse Reaction
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CV	Curriculum Vitae
cfDNA	Cell free deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EU	European Union
GCP	Good Clinical Practice
IC	Informed Consent
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
NGS	Next generation sequencing
NSCLC	Non-small cell lung cancer
PCR	Polymerase chain reaction
PK	Pharmacokinetics
(S)AE	(Serious) Adverse Event
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
SUSAR	Suspected Unexpected Serious Adverse Reaction
TKI	Tyrosine kinase inhibitor
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: In lung cancer patients with oncogenic driver mutations TKI are used as targeted therapy, but eventually acquired resistance will take place. Standard of care is to use invasive rebiopsy to diagnose resistance mechanisms, while new methods of testing on circulating tumor material could detect the mutations of interest in plasma. Possibly this means that detection can take place even before clinical progression is seen, without need of an invasive procedure. Pharmacokinetic differences may influence the development of acquired resistance.

Objective: To collect repeated samples of blood from patients (starting) on TKI, for liquid mutation testing, and pharmacokinetic analysis.

Study design: observational study with extra blood sampling at already planned moments of blood withdrawal.

Study population: Adult NSCLC patients treated with TKI.

Main study parameters/endpoints: primary mutation plasma levels, resistance mutation plasma levels, pharmacokinetics of TKI (through serum levels). Secondary endpoints: correlation of (resistance) mutation levels and pharmacokinetics to progression, and to biopsy specimen results when applicable.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The extra risk of taking three additional tubes during blood withdrawal is regarded negligible.

1. INTRODUCTION AND RATIONALE

Non-small cell lung cancer (NSCLC) has a poor prognosis, especially once metastasized.[1]

The last years increasing numbers of oncogenic driver mutations have been discovered and agents for targeted therapy have been developed, see figure 1. Although therapy efficacy is promising, metastasized cancer still cannot be cured, and all patients will show progression eventually despite targeted therapy. Several resistance mechanisms have been identified so far, see figure 2, but research on this subject is ongoing.

Pathologic investigation of histologic tumor tissue can detect resistance mutations and is considered the gold standard test, but this implicates an invasive procedure for tissue collection for patients. New techniques provide possibilities to detect mutations on circulating tumor DNA, like digital droplet polymerase chain reaction (PCR) and next generation sequencing (NGS). Besides the less invasive character (blood collection), it might also be faster in diagnosing acquired resistance to first line TKI by repeated measures than waiting for progression on radiologic investigation and taking a new biopsy. Also, this approach may provide the necessary information on tumor status when a tissue biopsy is not possible. The sensitivity of liquid biopsies in patients with metastasized mutated lung cancer is about 70% in literature.[2]

The best known oncogenic driver mutation is that of Epidermal Growth Factor Receptor (*EGFR*), present in 10-15% of cases in the Caucasian population up to 60% in the Asian population. This is providing possibilities for targeted therapy with high response rates and long progression free survival.[3] Although this population indeed shows a high response rate to first-line EGFR-tyrosine kinase inhibitors (EGFR-TKI), all patients show progression of disease due to acquired resistance eventually. The most frequent seen acquired resistance mechanism is p.T790M, a mutation which leads to inability of first/second generationEGFR-TKI to bind to the specific domain, which occurs in about 50% of cases.[4,5] For this mutation, a novel agent has been developed (osimertinib) that shows high respons rates and a new period of progression free survival.[6,7] Osimertinib has been registered for the indication of progression on first-line TKI in p.T790M positive patients. More recently osimertinib showed superior PFS and OS when administered upfront as first line therapy in *EGFR*-mutated NSCLC.[8,9] Research on resistance mechanisms when osimertinib is used in first line is ongoing.

A similar situation exists for Anaplastic Lymphoma Kinase (*ALK*)-translocated NSCLC patients, for which new agents that are active for acquired resistance mechanisms are investigated. The most common resistance mutation is p.L1196M.[10]

A group of rising interest consists of the *BRAF* mutant lung cancer patients. The most common activating mutation is p.V600E, which occurs in 1-3% of non-small cell lung cancers and is also known and targeted in melanoma. In 6-8% of NSCLC patients a *BRAF* mutation is found, both p.V600E and non-V600. Recently, promising results were

published on combined targeted treatment with dabrafenib and trametinib in both second line and first line.[11,12] This led to the FDA and EMA approval of the combination of these agents for advanced and metastatic p.V600E *BRAF* mutated NSCLC. The non-p.V600E mutations are also of interest, as recent research showed activity of these agents in non-V600E mutated cell models.[13] Also in the *BRAF* p.V600E mutated population resistance will take place, for which monitoring of the original mutation and detection of resistance mechanisms seem useful.

As research and clinical practice concerning oncogenic drivers and targeted therapies are developing in fast pace, we would like to be able to explore the plasma parameters in lung cancer patients with an activating mutation as soon as new TKI treatments are available for them. Therefore we will for example also include patients with KRAS p.G12C treated with AMG-510 (sotorasib)[14] when applicable, and patients with MET exon 14 skipping, ROS1 or NTRK fusions when treated with TKI (e.g. capmatinib[15], crizotinib[16] or entrectinib[17]) in clinical practice by their treating physician.
New cohorts of patients with specific genetic aberrations and treated with TKI will be formed and included according to developments in clinical practice.

We will correlate the results of the cell free DNA (cfDNA) liquid mutation detection techniques with the time to progression, and the response to other therapies after switching. Also, we will compare the results of the plasma tests with the results of a rebiopsy (planned by the treating specialist as standard of care) to determine the diagnostic value when applicable.

Pharmacokinetics (PK) might also play a role in acquiring resistance. Possibly the systemic exposure to the TKI influences the development of resistance of the tumor to the TKI. We will evaluate the differences in pharmacokinetics between responders, non-responders due to fast progression, and progression due to acquired resistance. Factors like body weight,
type of mutation and smoking status will be incorporated in the analysis. We will also relate PK to TKI effectiveness and toxicity.

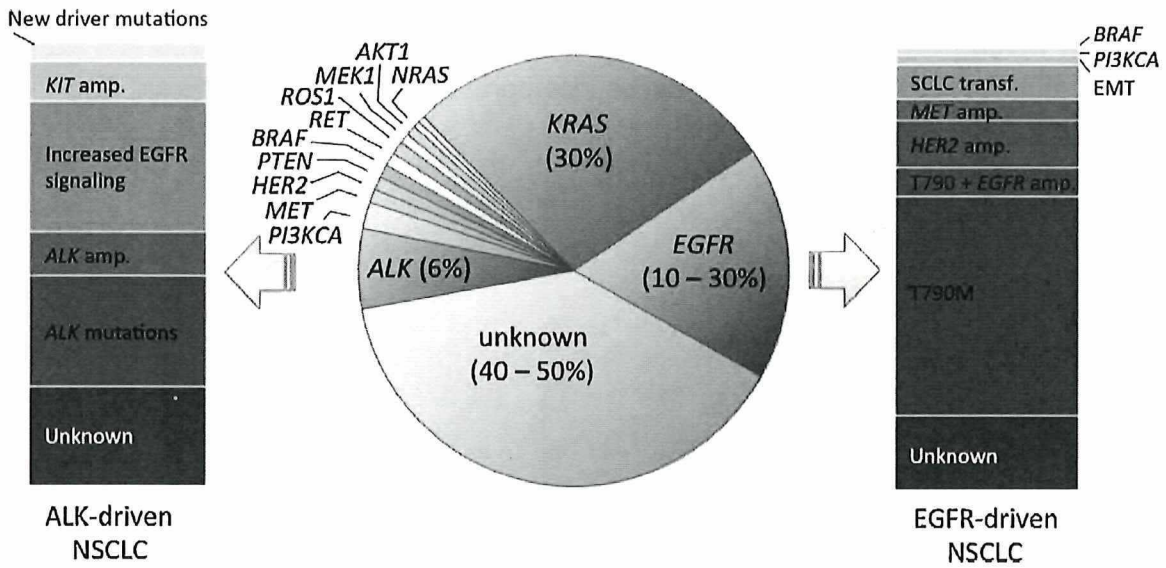
Figure 1. Situation in 2016 [18] , with currently even more treatment options available, see also https://www.mycancergenome.org/content/disease/non-small-cell-lung-carcinoma/ (accessed 22-10-2020)

Table 1. Frequency of Mutations and Availability of Targeted Therapies in NSCLC.

Gene	Alteration	Frequency in NSCLC
AKT1	Mutation	1%
ALK	Rearrangement	3–7%
BRAF	Mutation	1–3%
DDR2	Mutation	~4%
EGFR	Mutation	10–35%
FGFR1	Amplification	20%
HER2	Mutation	2–4%
KRAS	Mutation	15–25%
MEK1	Mutation	1%
MET[a]	Amplification	2–4%
NRAS	Mutation	1%
PIK3CA	Mutation	1–3%
PTEN	Mutation	4–8%
RET	Rearrangement	1%
ROS1[a]	Rearrangement	1%

Key:
Drugs approved in NSCLC.
Drugs approved in NSCLC but for other molecular subtype
Drugs approved in other cancer.
Drugs in clinical development.

Figure 2. Frequency of oncogenic driver mutations and resistance mechanisms on first line therapy in EGFR-mutated and ALK-rearranged NSCLC[19]



2. OBJECTIVES

Primary Objective: to explore (primary and resistance) mutation levels in blood at sequential time points, to identify mutation resistance mechanisms , and to explore TKI concentration levels in time.

Secondary Objective(s):

- To compare different plasma mutation detection methods (ddPCR, NGS)
- To correlate systemic and intratumoral pharmacokinetic parameters with progression free survival and toxicity, and to determine the influence of other variables (e.g. comedication, BMI and smoking status) on these pharmacokinetic parameters
- To correlate plasma mutation detection to biopsy mutation analysis performed by the treating specialist as standard of care when applicable

3. STUDY DESIGN

This is an observational study in pulmonary oncology patients treated with TKI.
Ideally before start of therapy, at week 4, 8 12, and then every 4-8 weeks (following the standard of care clinical pathways local guidelines) extra tubes of blood will be collected during blood withdrawal planned for standard-of-care. When a TKI switch takes place, patients receive a new study number and the blood collection will be continued following standard-of-care. Sampling will be performed during steady-state in working hours before next ingestion of TKI (the regular morning dose will need to be postponed until after blood withdrawal).
The moment of blood withdrawal after start of therapy and after last dosage of TKI will be registered.
At the time of progression the current standard of care is to perform a rebiopsy, to identify the resistance mechanism and determine the next appropriate systemic therapy for a patient. Some mechanisms can only be determined on a biopsy specimen (e.g. transformation to SCLC, MET amplification FISH). This study is observational and will not interfere with the current standard practice, therefore the treating physician is free to determine the indication for and possibility of a rebiopsy. However, when a biopsy is taken as standard of care, we will also draw an extra blood sample on the day of the biopsy (preferably during standard preprocedural coagulation status laboratory investigations) and use a portion of the biopsy for a fresh frozen specimen, as evolving molecular investigations (like RNA analysis) often need fresh non-fixed material to obtain reliable results.

Four core biopsies will be obtained for further analysis. Half of the obtained biopsy material will be stored in formalin according to local standard procedures, while the other half will be fresh frozen according to local standard procedures and stored for further analysis.
When the treating physician needs to take additional tissue or liquid (e.g. pleural fluid, liquor) for investigations following standard-of-care protocols, we would like to perform additional molecular and/or pharmacokinetic analysis on the residual material when applicable.

4. STUDY POPULATION

4.1 Population (base)

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Age ≥ 18 years
- Able to understand the written informed and able to give informed consent
- Locally advanced or metastatic NSCLC with oncogenic driver mutation
- Treatment with TKI according to standard of care

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Unable to draw blood for study purposes

4.4 Sample size calculation

A sample size calculation on beforehand is not possible because of the exploratory character of this study. A null hypothesis could not be formulated.
We aim to identify a relation between levels of mutation and TKI concentrations in plasma and progression. We aim to include a minimum of 50 patients per mutation and TKI category (e.g. EGFR mutation on erlotinib therapy, on osimertinib therapy etc). This number is necessary to collect 30 positive blood samples in each category, assuming the 70% sensitivity of liquid biopsies in literature and experienced 15% dropout (due to withdrawal of consent, or medication switch for other reasons than progression, e.g. toxicity). When exploring the relation between other mutation levels and progression free survival (e.g. co-occurring mutations), we noticed that the population size was quite small to identify a significant correlation (manuscript published in Cancers[20]), therefore the group would ideally increase to 100 patients (to collect 60 positive blood samples for analysis).

5. TREATMENT OF SUBJECTS

Treatment will be performed according to standard of care, i.e. evaluation of treatment effect, toxicity and other clinical parameters will be performed by the treating physician according to local practice. None of the study procedures will influence decision making in the treatment.

5.1 Investigational product/treatment

Not applicable

5.2 Use of co-intervention (if applicable)

Not applicable.

5.3 Escape medication (if applicable)

Not applicable.

6. INVESTIGATIONAL PRODUCT

Not applicable.

7. NON-INVESTIGATIONAL PRODUCT

Not applicable.

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

Resistance and primary mutation plasma levels (e.g. *EGFR* p.T790M and *ALK* p.L1196M, absolute and relative count), and systemic TKI concentrations in time

8.1.2 Secondary study parameters/endpoints (if applicable)

Time to progression or death.
Pharmacokinetics of intratumoral TKI (through levels)Primary mutation levels by different mutation detection methods
Correlation of mutation status in blood to (re)biopsy specimen results performed for standard-of-care.
Effect of other variables (e.g. comedication, smoking status and BMI) on PK parameters.

8.2 Randomisation, blinding and treatment allocation

Not applicable.

8.3 Study procedures

8.3.1 Registration

The informed consent form must be personally signed and dated by all patients before any study specific procedure is performed. The registration will be performed by one of the study coordinators or their delegates at the above mentioned contact address. Study ID's will be assigned in chronological order.

8.3.2 Blood withdrawal

Patients have to be treated with TKI for a driver mutation to be eligible for this study. Since regular blood withdrawal is necessary for standard of care in these patients, blood collection for samples can be performed during these already planned moments. In this way, there is no need for extra invasive investigations for subjects.

8.3.3 Liquid testing sample

Two 10mL CellSave Preservative vacutainer tubes (or a comparable alternative when applicable) are filled and processed following instructions in the appendices.

8.3.4 Pharmacokinetic sample

A 4 mL lithium-heparin vacutainer tube will be drawn, stored on ice and centrifuged within 30 minutes for 10 minutes at 2500g and 4°C. Aliquots of 500uL will be stored at least at -20°C. Blood samples will be analysed using a validated LC-MS method at

the Laboratory of Translational Pharmacology in the Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands.

8.3.5 Clinical Record Forms

Clinical parameters of each patient will be recorded anonymously in the main study file. The file will contain the following sections:

- Baseline: patient demographics
- Cancer history: diagnosis, history of treatment
- Treatment: dose, date of start, date of interruption if applicable
- Toxicities: adverse events of CTCAE grade 3 or higher
- Outcome: all assessments for evaluation of treatment effect as performed for standard of care

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.4.1 Specific criteria for withdrawal (if applicable)

Not applicable

8.5 Replacement of individual subjects after withdrawal

Not applicable.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable.

8.7 Premature termination of the study

Since patients are treated according to standard of care and the results of the test do not influence clinical decision-making, no premature termination of the study is foreseen.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardize subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the blood draw. All adverse events as a consequence of the blood withdrawal reported spontaneously by the subject or observed by the investigator or his staff will be recorded. Other AE's will be recorded to explore toxicity correlation to TKI concentrations.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

The investigator will report all SAEs as a consequence of the blood withdrawal to the sponsor without undue delay after obtaining knowledge of the events, except for the following SAEs: SAEs not related to the blood withdrawal.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for

SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable

9.3 Annual safety report

Not applicable

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

9.5 Data Safety Monitoring Board (DSMB) / Safety Committee

Not applicable

10. STATISTICAL ANALYSIS

10.1 Primary study parameter(s)

The relative presence of plasma mutations, and actual concentrations of TKI will be described over time (until progression). The relation to progression free survival will be explored by Kaplan Meier survival analysis.

To determine a clinical relevant cutoff value, the relation of time to progression and mutation levels will be explored, by means of survival analysis methods.

We will determine the clinical relevant cutoff value (both absolute and relative values) with the last measurement before radiological progression (≤6 weeks to progression), because we consider it relevant to know the moment of progression one outpatient visit earlier than radiologic progression is visible. In case of a missing value, we will carry forward the last known value if time since last known value ≤12 weeks.

10.2 Secondary study parameter(s)

We will explore the relation between the mean TKI level and the time to reach the clinical relevant cutoff value by means of Cox regression analysis. Also we will evaluate if the PK parameters are different in groups with different PFS and cfDNA levels. Effects of BMI and smoking status on PK will be explored by means of regression analysis.

Diagnostic value of plasma mutation detection will be evaluated using different techniques (ddPCR and NGS) and rebiopsy specimen results when performed for standard-of-care.

Concordance of detection of tumor mutations will be evaluated between ddPCR, NGS in blood, and NGS in tissue samples when available.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

The investigator will ensure that this study is conducted to the standards of Good Clinical Practise, in full concordance with the 'Declaration of Helsinki' (latest amendment http://www.wma.net/en/30publications/10policies/b3/), the Dutch laws and regulations with the WMO ('Wet Medisch-wetenschappelijk Onderzoek met mensen') in particular.

11.2 Recruitment and consent

Patients with NSCLC and an oncogenic driver mutation will be asked to participate in this study by their treating medical specialist. When they are interested to participate they will receive further explanation and information, and also written information (informed consent form). Because filling of extra tubes during regular blood drawing is the only study related intervention, patients are also allowed to sign the ICF immediately, after careful reading. However, a period of consideration is also allowed. The ICF must be signed by the patient and the person who has conducted the informed consent procedure before blood drawing. The patient will be provided with a second original of the signed informed consent statement. The subject may withdraw from the study at any time, without stating their reasons, and without prejudicing future medical treatment.

11.3 Objection by minors or incapacitated subjects (if applicable)

Not applicable.

11.4 Benefits and risks assessment, group relatedness

All blood samples will be collected during an already necessary moment of blood drawing, on the outpatient clinic. Since this is a moment for standard of care, the extra risk of the study procedures is negligible.

11.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.

The METC Erasmus MC has given exemption for the WMO-proefpersonenverzekering, because participating in the study is without extra risks

11.6 Incentives (if applicable)

Not applicable

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Each patient will be given a unique patient study number. All data and human materials collected from patients will be stored for 15 years. Any rest materials will be destroyed at the end of the study, unless the subject has given consent on the ICF to store rest materials for a maximum of 15 years. Study data will be accessed by authorized personnel only.

12.2 Monitoring and Quality Assurance

This is a protocol with negligible risk and the study will be monitored accordingly.

Monitoring of the study will take place once a year and will be performed by an independent, qualified research collaborator designated by the research Centre of the pulmonary department in the Erasmus MC. The monitor will monitor the documents that are minimally required according to the monitoring plan of the Erasmus MC.

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

12.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

We will submit trial results for publication in a peer reviewed scientific journal.

13. STRUCTURED RISK ANALYSIS

Not applicable

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Appendix A

Method Blood collection for plasma cfDNA isolation
Date: July, 2016
Authors: Maurice Jansen, John Martens (Department of Medical Oncology, Erasmus MC – Cancer Institute, Rotterdam, The Netherlands)

Pre-analytical conditions
After obtaining written informed consent, at least 2x10 mL of blood samples are collected within a single blood draw, in 10 mL CellSave Preservative (Janssen Diagnostics, Raritan, NJ, USA) vacutainer tubes. Shipment of blood is at room temperature. The blood samples will be processed for plasma isolation within 96 hours after blood draw. Plasma is isolated using 2 sequential centrifugation steps: 1) 1711*g* for 10 minutes at room temperature; 2) 12,000*g* for 10 minutes at 4°C. Plasma is stored at -80°C in 1 ml aliquots immediately after centrifugation until further processing.

cfDNA isolation and quantification
For cfDNA isolation plasma samples are thawed at 4°C and 3 ml of plasma per sample is used. cfDNA is isolated using the QIAamp® Circulating Nucleic Acid kit (QIAGEN, Venlo, Limburg, The Netherlands) according to manufacturer's instructions. cfDNA is eluted from the QIAGEN® Mini column using 50 µL buffer AVE and is applied 3 times to the column to obtain the highest cfDNA concentration possible. cfDNA is stored at -20°C. cfDNA concentrations are quantified using the Quant-iT dsDNA high-sensitivity assay (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and the Qubit fluorometer (Invitrogen) is used as readout.