PREDICTION PROTOCOL

Response and Toxicity Prediction by Microbiome analysis in locally advanced NSCLC treated with IO (durvalumab, MEDI4736) after Chemoradiotherapy

PROTOCOL TITLE: Response and Toxicity Prediction by Microbiome analysis in locally advanced NSCLC treated with IO (durvalumab) after chemoradiotherapy.

Protocol ID	NL76017.058.21
Short title	PREDICTION
EudraCT number	Not applicable
Version	6.0
Date:	March 17, 2023
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Multicenter research: per site	LUMC, Leiden AVL/NKI, Amsterdam Jessa Hospital, Hasselt, Belgium AZ Turnhout, Turnhout, Belgium AZ Delta, Roeselare, Belgium OLV Aalst, Aalst, Belgium
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Subsidising party	AstraZeneca
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Laboratory sites	TNO (microbioom analysis LUMC Pulmonology (blood analysis)
Pharmacy	Not applicable

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)**
AE	**Adverse Event**
ALK	**anaplastic lymphoma kinase**
ALT	**alanine aminotransferase**
ANC	**absolute neutrophil count**
aPPT	**activated partial thromboplastin time**
AST	**aspartate aminotransferase**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CRT	**chemoradiotherapy**
CRF	**case report form**
CT	**computed tomography**
CTLA-4	**cytotoxic T-lymphocyte-associated protein 4**
CV	**Curriculum Vitae**
CyTOF	**cytometry by time of flight**
DCR	**disease control rate**
EU	**European Union**
ECOG	**eastern cooperative oncology group**
EGFR	**epithelial growth factor receptor**
GCP	**Good Clinical Practice**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
GFR	**glomerular infiltration rate**
HCV	**hepatitis C virus**
IC	**Informed Consent**
ICI	**Immune Checkpoint Inhibitors**
INR	**international normalized ratio**
irAE	**immune-related adverse event**
METC	**Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)**
NK	**natural killer**
NSCLC	**non-small cell lung cancer**
PFS	**progression free survival**
PD	**pharmacodynamic**
PD-L1	**programmed death-ligand 1**
PT	**prothrombin time**
RNA	**ribonucleic acid**
(S)AE	**(Serious) Adverse Event**
SOC	**standard of care**

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**
TPS	**tumor proportion score**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
ULN	**upper limit of normal**
VOC	**Volatile Organic Compounds**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale:
To investigate the role of the microbiome in response and toxicity prediction in patients with locally advanced NSCLC treated with durvalumab after concurrent or sequential chemoradiotherapy.

Objective:
The predictive value of the microbiome (throat swabs and stool samples) to identify patients who will relapse during standard durvalumab treatment after chemoradiotherapy at 6 months. Exploratory endpoints include the effects of antibiotic therapy before and during durvalumab treatment on toxicity and clinical outcome.

Study design:
Prospective observational multicenter study.

Study population:
Patients ≥ 18 years with non-small cell lung cancer who will start treatment with durvalumab after completion of chemoradiotherapy.

Intervention:
Not applicable

Main study parameters/endpoints:
To investigate if the microbiome (throat swabs and stool samples) can be used to identify patients who will relapse during durvalumab treatment after chemoradiotherapy at 6 months.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:
Collection of throat swabs and stool samples before start of durvalumab treatment; sampling of blood. No benefit is foreseen in participation to this study.

1. INTRODUCTION AND RATIONALE

The development of antibodies targeting the programmed death 1 (PD-1) axis and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) provided a major clinical improvement in treatment across tumor types. These drugs promote antitumor immunity by blocking inhibitory immune pathways, often resulting in tumor regression and improved survival. In advanced metastatic non–small-cell lung cancer (NSCLC) patient selection, based upon PD-L1 tumor proportion score (TPS) and tumor mutational burden (TMB), moved PD-1 targeted immunotherapy from further lines to frontline therapy in monotherapy, bitherapy or combined with chemotherapy, showing a consistent more favorable response over conventional chemotherapy [1][2][3][4][5][6][7][8].

Improved clinical outcomes after adjuvant treatment with durvalumab following chemoradiotherapy (CRT) for locally advanced NSCLC (PACIFIC trial program) [9][10][11], led to the rapid adoption of this treatment strategy as standard of care. However, despite the improved progression free survival and overall survival, recurrence rate remains high. Approximately 45% of patients will relapse within 1 year, despite adjuvant durvalumab therapy. To date no performant biomarker predicting treatment response or failure nor toxicity exists and the number of prospective studies addressing this issue is limited. Both PD-L1 TPS and TMB should be considered ‘enriching’ parameters improving response-chances, but they are far from an ideal biomarker. Non-invasive biomarkers are essential in the future for better patient selection and therapy allocation. One of the potential non-invasive biomarkers of interest is the microbiome.

The microbiome gained a lot of interest as a potential biomarker the past years. Multiple studies demonstrated that the gut microbiota modulates the response to inhibitors of PD1-PDL1 axis [12][13][14][15]. Overall, they indicate that a “healthier”, highly diverse microbiota and the presence of certain bacterial species favor the establishment of an anti-tumor immune response at baseline that may be enhanced by the anti-PD1 treatment with favorable clinical response. Alteration of microbiota balance by antibiotics treatment near the initiation of the therapy reduces its efficacy.

1.1 Pre-clinical Trial Data

In the PACIFIC study, 709 patients who were randomized to receive consolidation therapy (473 received durvalumab and 236 received placebo) were included. The median progression-free survival from randomization was 16.8 months ([CI], 13.0 to 18.1) with durvalumab versus 5.6 months (95% CI, 4.6 to 7.8) with placebo. At 6 and 12 months there was a superior outcome for the durvalumab arm. At 6 months the percentage of patients who progressed was 32% versus 52% for placebo. At 12-month the progression-free survival rate was 55.9% versus 35.3%. The response rate was higher with durvalumab than with placebo (28.4% vs. 16.0%; P<0.001), and the median duration of response was longer (72.8% vs. 46.8% of the patients had an ongoing response at 18 months). The median time to death or distant metastasis was longer with durvalumab than with placebo (23.2 months vs. 14.6 months; P<0.001). Grade 3 or 4 adverse events occurred in 29.9% of the patients who received durvalumab and 26.1% of those who received placebo; the most common adverse event of grade 3 or 4 was pneumonia (4.4% and 3.8%, respectively). A total of 15.4% of patients in the durvalumab group and 9.8% of those in the placebo group discontinued the study drug because of adverse events. Clinical benefit however could be obtained without compromising patient reported outcomes [16].

Microbiome data: An eloquent study involving mice subcutaneously injected with melanoma (B16) and colon carcinoma (MC38) cells pretreated with an antibiotic cocktail was among the first to show the relationship between the stool microbiome and response to immunotherapy [17]. Antibiotic-treated and germ-free mice showed significantly shorter survival and less tumor volume reduction with immunotherapy (through injections of CpG-oligonucleotides and anti-interleukin (IL)-10 antibodies), when compared to controls, and highlighted that commensal gut microbiota primed tumor-infiltrating myeloid-derived cells through Toll-like receptor 4 (TLR4) activation and produce cytokines such as tumor necrosis factor (TNF) critical to antitumor efficacy. Notably, administration of cultured *Allstipes species (spp., A. shahii)* or *Lactobacillus spp*. by gavage reconstituted or attenuated TNF-dependent tumor response to immunotherapy in antibiotic-treated mice, respectively. Numbers of *Lactobacillus spp*. recovered as early as 1 week after stopping antibiotics, but recovery of *Allstipes* and *Ruminococcus spp.* was delayed, taking up to 4 weeks after stopping antibiotics.

Single cell analyses by mass cytometry: Systems immunology is a relatively new field that aims at better understanding the immune system as a whole, and at identifying cells, molecules and pathways involved in particular pathological settings [18] [19]. Systems biology approaches to immunology combine cutting-edge techniques, including single-cell transcriptomics and mass cytometry with bioinformatics, for the analysis of multidimensional data [20][21][22]. Mass cytometry, also called cytometry by time-of-flight (CyTOF), is a variation of flow cytometry, in which antibodies are labelled with heavy metal ion tags rather than fluorochromes, the readout being by time-of-flight mass spectrometry. This allows for the combination of many more antibody specificities in a single sample, without significant spillover between channels. The lack of any significant mass spectral overlap and autofluorescence background makes mass cytometry uniquely suited for complex high-dimensional phenotypic and functional analysis at the single cell level.

Despite the improvements in overall and progression free survival in patients treated with chemoradiotherapy followed by consolidation therapy with PD-L1 blockade (durvalumab), a significant number of patients suffer from recurrent disease within one year.
To allow for a more adequate patient follow-up, identification of a biomarker predicting early recurrence or long-lasting disease control is required. The former group could be monitored more closely while the latter can suffice with less outpatient visits and less radiological evaluations. Ineffective therapies can be ceased earlier and allow a switch to a more effective regimen. This seems appropriate since assessment of tumor recurrence after CRT is more complicated due to the ample possibility of non-malignant changes in the lung (radiation pneumonitis or infection). So far, no biomarkers have been identified for this purpose.
Recently, the microbiome has attracted attention for its ability to modify the immune system and thereby changing the immune surveillance in humans. This is considered to be of pivotal importance to sustain a long-lasting tumor control in different tumor types treated with immune checkpoint inhibitors.

There is an urgent need to improve the survival in patients who progress early during adjuvant immunotherapy (IO) therapy after CRT for NSCLC. The standard of care (SOC) is based of clinical and radiological changes which can be difficult to interpret (see 4.2.1). The microbiome may be able to identify patients at risk for early relapse or development of toxicity. By identifying these patients, we might be able to improve the clinical outcome.

2. OBJECTIVES

2.1 Primary objective

Primary objective: To investigate the predictive value of the microbiome (throat swabs and stool samples) to identify patients who will relapse during durvalumab treatment after chemoradiotherapy at 6 months.

Hypothesis: In approximately 32% of patients a tumor relapse will occur within 6 months after completion of chemoradiotherapy despite adjuvant durvalumab therapy. The microbiome plays a key role in the modulation of the immune system. Using the microbiome as non-invasive biomarker we will aim to identify those patients at risk of an early recurrence.

2.2 Secondary Objectives

1. To investigate the predictive value of the microbiome (throat swabs and stool samples) to identify patients that develop immune-related adverse event (irAE's) during durvalumab treatment after chemoradiotherapy.

   Hypothesis; The microbiome plays a pivotal role in modulating host immunity and has been suggested to be a major determinant of immunotherapy-related toxicity. Using the microbiome as a non-invasive biomarker we will try to identify those patients at risk for developing irAE's.

2. To determine disease control rate (DCR ie. stable disease, partial response, and complete response) and PFS at 6 months after completion of chemoradiotherapy and correlate this to the microbiome samples.

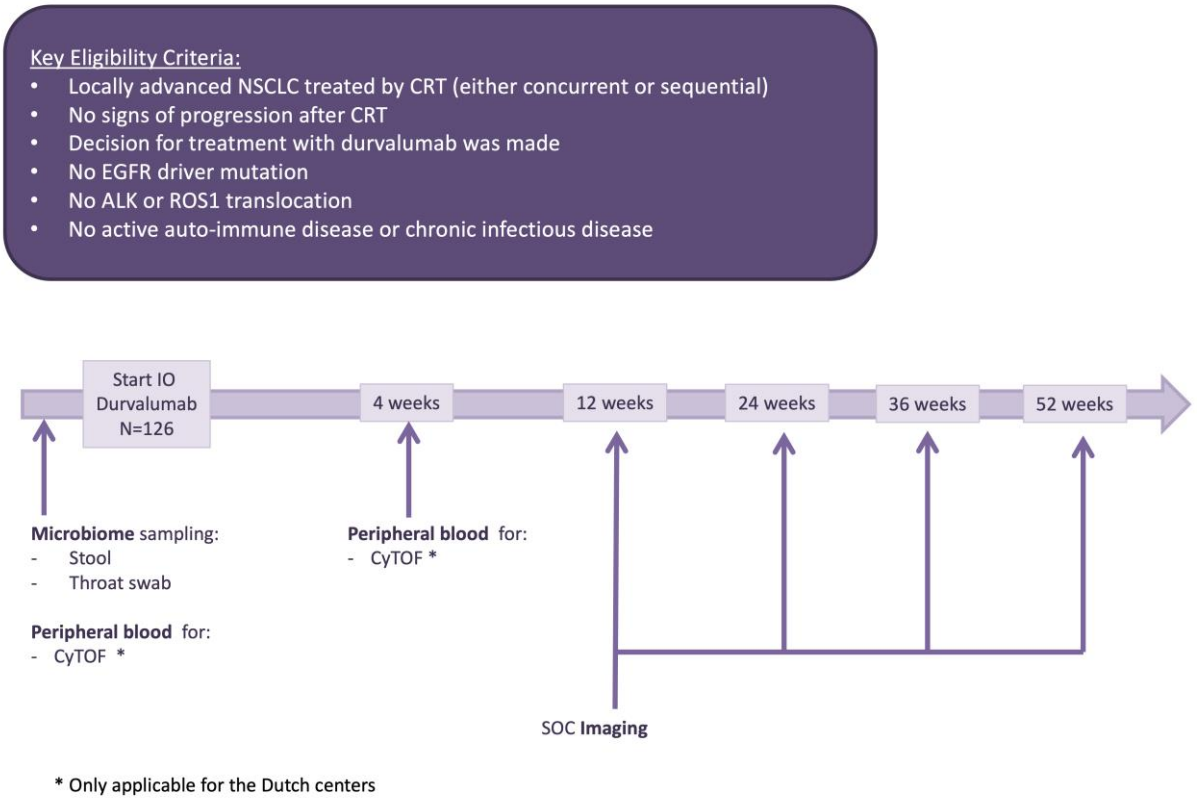
   Hypothesis: Durvalumab consolidation after chemoradiotherapy for locally advanced NSCLC was able to improve DCR and PFS. We will try to identify patients that benefit from durvalumab consolidation using the microbiome as a non-invasive biomarker.

3. To investigate to what extend cytological characteristics of circulating immune cells obtained from responders and non-responders differ and to explore to what extend these differences relate to the microbiome.

   Hypothesis: The changes in microbiota composition of the stool and throat will correlate with the phenotype of immune cells observed by mass cytometry.

3. STUDY DESIGN

This is a prospective observational multicenter study, during 2 years after first patient in, 126 patients will be included. Patients be included from 2 centers in the Netherlands and 4 centers in Belgium.



4. STUDY POPULATION

4.1 Population (base)

Patients ≥ 18 years with non-small cell lung cancer stage III who will be treated with durvalumab as per standard of care after completion of concurrent or sequential chemoradiotherapy.

4.2 Inclusion criteria

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

1. Stages IIIA, IIIB and IIIC (as per UICC 8th TNM edition) NSCLC (histologically or cytologically confirmed) who will be treated with durvalumab treatment after concurrent or sequential chemoradiotherapy according to local standards. Patients that received neoadjuvant/adjuvant chemotherapy for surgically treated stages I to III NSCLC are allowed as long as therapy was completed at least 6 months prior to the diagnosis of disease recurrence amenable for CRT and resolution of all treatment related toxicity ≤ grade 1.

2. No signs of disease progression after chemoradiotherapy

3. ≥ 18 years

4. ECOG ≤ 1

5. Must be willing to provide collected stool samples and allow to obtain a throat swab during the observation period.

6. Demonstrate adequate organ function as defined in Table 1, all screening labs should be performed within 10 days of start durvalumab

Table 1: Adequate Organ Function Laboratory Values

System	Laboratory Value
Hematological	
Absolute neutrophil count (ANC)	≥1,500 /mcL
Platelets	≥100,000 / mcL
Hemoglobin	≥9 g/dL or ≥5.6 mmol/L
Renal	
Serum creatinine OR Measured or calculated creatinine clearance* (GFR can also be used in place of creatinine or CrCl)	≤1.5 X upper limit of normal (ULN) OR ≥50 mL/min for subject with creatinine levels > 1.5 X institutional ULN
Hepatic	
Serum total bilirubin	≤ 1.5 X ULN OR
	Direct bilirubin ≤ ULN for subjects with total bilirubin levels > 1.5 ULN
AST (SGOT) and ALT (SGPT)	≤ 2.5 X ULN OR ≤ 5 X ULN for subjects with liver metastases
Coagulation	
International Normalized Ratio (INR) or Prothrombin Time (PT)	≤1.5 X ULN unless subject is receiving anticoagulant therapy as long as PT or PTT is within therapeutic range of intended use of anticoagulants
Activated Partial Thromboplastin Time (aPTT)	≤1.5 X ULN unless subject is receiving anticoagulant therapy as long as PT or PTT is within therapeutic range of intended use of anticoagulants
*Creatinine clearance should be calculated per institutional standard.	

4.3 Exclusion criteria

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

1. Is currently participating in or has participated in a study of an investigational agent or using an investigational device within 4 weeks of the first dose of treatment.

2. Has had prior monoclonal antibody therapy within 4 weeks prior to study Day 1 or who has not recovered (i.e., ≤ Grade 1 or at baseline) from adverse events due to agents administered more than 4 weeks earlier.

3. Previous treatment with PD-1-PD-L1 axis inhibiting immunotherapy.

4. Active or history of autoimmune disease or immune deficiency, including, but not limited to, myasthenia gravis, myositis, autoimmune hepatitis, systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, anti-phospholipid antibody syndrome, Wegener granulomatosis, Sjögren syndrome,

Guillain-Barré syndrome, multiple sclerosis, vasculitis, or glomerulonephritis with the following exceptions:

- Patients with a history of autoimmune-related hypothyroidism who are on thyroid-replacement hormone are eligible for the study.
- Patients with controlled Type 1 diabetes mellitus who are on an insulin regimen are eligible for the study.
- Skin disorders (such as vitiligo, psoriasis, or alopecia) not requiring systemic immunosuppressive treatment (including corticosteroids) are permitted to enrol.

5. Subjects with a condition requiring systemic treatment with either corticosteroids (> 10 mg daily prednisone equivalents) or other immunosuppressive medications within 14 days of start of Durvalumab. Inhaled or topical steroids, and adrenal replacement doses > 10 mg daily prednisone equivalents are permitted in the absence of active autoimmune disease.

6. Subjects who have undergone organ transplant or allogeneic stem cell transplantation.

7. Active malignancy or a prior malignancy within the past 3 years, with the following exceptions:
   - Patients with completely resected basal cell carcinoma, cutaneous squamous cell carcinoma, cervical carcinoma in-situ, breast carcinoma in-situ, and patients with isolated elevation in prostate-specific antigen or low risk prostate cancer (managed with watchful waiting) are allowed.
   - Patients who underwent mediastinal radiotherapy in the past are not allowed.

8. Subjects with known chronic infections/infectious disorders (eg. *Clostridium colitis*)

9. Have known but untreated driver mutations of the EGFR gene or ALK or ROS1 translocation.

10. Has evidence of symptomatic interstitial lung disease or an active, non-infectious pneumonitis.

11. Has an active chronic infection requiring systemic therapy.

12. Has a history or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the trial, interfere with the subject's participation for the full duration of the trial, or is not in the best interest of the subject to participate, in the opinion of the treating investigator.

13. Has known psychiatric or substance abuse disorders that would interfere with cooperation with the requirements of the trial.

14. Has a known history of Human Immunodeficiency Virus (HIV) (HIV 1/2 antibodies)

15. Has known active Hepatitis B (e.g., HBsAg reactive) or Hepatitis C (e.g., HCV RNA [qualitative] is detected).

16. Has received a live vaccine within 30 days prior to the first dose of trial treatment.

4.4 Sample size calculation

The primary goal of the study is identify patients, that develop disease progression or relapse (PD) within 6 months during adjuvant durvalumab after chemoradiotherapy, by the composition of the gut and throat microbiome as measured by the alpha and beta components (see 7.1). These are defined as a group of certain species and the distance (overlap) of the cultured species. Since there are no well-defined data for the microbiome available and a search for the predictive value of any one of the thousand different bacteria species is in vain, we summarize the data using two diversity measures; the alpha and beta component according to the Shannon-Weaver index [23].

Formal power analysis and sample size calculation is difficult to perform within these premisses. Sample size is based on data from previous studies. These studies examined the microbiota composition in adults with different cancer types. Some studies were explorative of nature and correlated with OS or PFS. These studies were summarized in Table 2 (adapted from [24]).

The purpose of our study is to train a classifier that will be validated in a subsequent study if it is promising enough. We will measure the predictive ability of the classifier on the basis of sensitivity and specificity, using cross-validation.
We expect at 6 months to identify 41 patients with progressive disease and 85 with non-progressive disease. We consider this study positive when the cross-validated sensitivity and specificity both lie entirely above 70% (one-sided 95% confidence intervals around both). With above numbers, this is achieved when minimally 29 out of 41 PD-patients and minimally 60 out of 85 non-PD patients are correctly identified, corresponding to an observed sensitivity of at least 82% and an observed specificity of at least 79% in our study population.

Table 2

Metabolites	N	Disease	ICIs	Effects	Reference
SCFAs - Fecal acetate (high) - Fecal proprionate (high) - Fecal butyrate (high) - Fecal Valeric Acid (high) - Plasme isovaleric acid (high)	52	Solid Cancers	Anti-PD-1	Longer PFS	[25]
SCFAs - Fecal proprionate (high) - Fecal butyrate (high) Fecal Lysine (high) Plasme Nicotinic acid (high)	11	NSCLC	Anti-PD-1	Longer Response	[26]
SCFAs - Fecal butyrate (high)	40	Melanoma Prostate Cancer	Anti-CTLA-4	Shorter PFS	[27]
SCFAs - Plasma proprionate (high)	45	Melanoma	Anti-CTLA-4	Shorter PFS	[27]
Serum kynurenine/tryptophan ratio (high)	106	Melanoma Renal cell carcinoma	Anti-PD-1	Shorter OS	[28]
3-Hydrozyanthranilic Acid (low)	19	NSCLC	Anti-PD-1	Longer PFS	[29]
Fecal 2-pentanone (high) Fecal Tridecane (high)	11	NSCLC	Anti-PD-1	Early progression	[26]
Serum indoleamine-2,3-diozygenase	23	NSCLC	Anti-PD1	ICI resistance	[30]

5. METHODS

5.1 Study parameters/endpoints

5.1.1 Main study parameter/endpoint

Progression of disease defined by iRECIST criteria on regular CT scan at 6 months following chemoradiotherapy.

5.1.2 Secondary study parameters/endpoints

Changes in circulating immune cells.

Occurrence of immunotherapy-related toxicity within 12 months following chemoradiotherapy.

5.2 Study procedures

Details regarding laboratory procedures/assessments to be performed in this trial are according to the local policies.

Stool samples and a throat swipe will be obtained using the recommended procedure developed by the TNO. See Appendix 1.

The CyTOF analysis will be performed on the blood drawn at baseline and in week 4. The total amount of blood to be drawn/collected for correlative studies over the course of the trial is 2 x 20 ml. The sampling will be performed only in the LUMC and AVL/NKI.See Appendix 2.

Pulmonary function test: Patients will perform hospital-based lung function measurements in accordance with the ERS/ATS criteria; Forced vital capacity (FVC), Forced Expiratory Volume in 1 second (FEV1) and diffusion capacity of the lung for carbon monoxide (DLCOc) will be measured.

See table 3 on the next page for an overview of all procedures, both Standard of Care and study-specific.

Table 3: Schedule of assesments

	Screening Phase	**During IO treatment (weeks)**				
	Main Study Screening (Baseline)	4	12	24	36	52
Informed Consent	x					
Inclusion/Exclusion Criteria	x					
Smoking status (active/recent vs hardly/never)	x					
Demographics and Medical History	x					
Antibiotics use review	x	x	x	x	x	x
Progression Status			x	x	x	x
Review Adverse Events	x	x				
Review immune related toxicity		x	x	x	x	x
Physical Examination	x			x		x
Vital Signs and Weight	x			x		x
ECOG Performance Status	x			x		x
Tumor Imaging (CT scan within +/- 2 wks)	x		x	x	x	x
Stool sampling*	x					
Throat swabs*	x					
Blood Samples (CyTOF) * #	x	x				
Pulmonary Function test (FVC, FEV1, F/V, TLCO)	x			x		x

*Only those items with an * are considered not the SoC procedures*
Item # is only applicable for the Dutch centers

5.3 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.

5.4 Replacement of individual subjects after withdrawal

Patients will be replaced when withdrawn from the study. Due to the low burden of this study, it is not expected that patients will withdraw.

5.5 Premature termination of the study

In case of slow accrual (<25 patients entered in the first year) the study can be terminated prematurely. In this scenario, the METC will be notified within 15 days.

6. SAFETY REPORTING

6.1 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 jeopardise 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.

6.2 AEs, SAEs and SUSARs

6.2.1 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 trial procedure. Only adverse events related to the collection of stool sampling, throat swabs and blood sampling will be recorded for this study.

6.2.2 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.

In case a subject is considered to be harmed by the collection samples of stool or blood the PI will be notified and discussed if the study must be adjusted or ended.

Only study related (S)AE's will be reported. This implies that all (S)AE's related to participation in this study protocol, meaning from stool sampling, throat swabs and blood sampling will be reported. The local investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events.

Immune related toxicity (so called immune related adverse events or irAEs) will be managed according to the local standard practice and recorded in the patient file.

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.

6.3 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, as defined in the protocol.

7. STATISTICAL ANALYSIS

SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) and MatLab (2017B, MathWorks, Natick, MA) will be used for data analysis. Descriptive statistics will expressed as mean ± SD if data are normally distributed and as median (interquartile range) if data are non-normally distributed. Between-group comparisons will be carried out using Mann–Whitney U tests, two-sample unpaired t-tests or chi-square tests. Within group comparisons will be carried out with paired T-test or in case of non-normal distribution with a Wilcoxon Signed Rank Test. Associations will be analyzed by (multiple) regression analysis.

7.1 Primary study parameter(s)

The primary goal of the study is to identify patients that develop disease progression or relapse (PD) within 6 months during adjuvant durvalumab treatment by the composition of the gut and throat microbiome as measured by the alpha and beta components.

Microbiota composition is expressed in a table of sequence reads, clustered at single nucleotide variants, each assigned to microbial taxa at various taxonomic levels. This will be used to establish possible differences in terms of (1) microbial (alpha) diversity, using the Shannon index and/or Simpson-index, (2) compositional differences (beta diversity), such as the Bray Curtis and/or UniFrac distances between samples, and (3) differential abundances of specific microbial taxa within the microbial community. Data might be further evaluated using principle component analysis and/or principal coordinate analysis.

7.2 Secondary study parameter(s)

Explorative analysis for relationships between immune-related toxicity (irAEs) during durvalumab treatment after chemoradiotherapy and composition of the microbiome (throat swabs and stool samples) will be done by (multiple) regression analysis. CT scans, lung function and other relevant clinical data will be used to identify irAE's.

Disease control rate (DCR ie. stable disease, partial response, and complete response) will be determined by CT scans and PFS at 6 months after completion of chemoradiotherapy. This will be correlated to the microbiome samples using multiple regression analysis.

In addition, CyTOF analyses will be tested for their usefulness in predicting these progressors. These are of pure experimental value.

8. ETHICAL CONSIDERATIONS

8.1 Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (version 2013). We consider this study to be inside the boundaries of the Medical Research Involving Human Subjects Act (WMO). The coordinator will implement the research project in full respect of (inter-)national legal and ethical codes of practice. All study documents, including the full protocols and informed consent forms and any additional materials will be submitted to the relevant national authorities. Studies will commence upon approval.

All information used in this study will be collected for routine clinical practice and will be handled, accessed and used according to the current regulatory framework, including the AVG. Appropriate mechanisms for data handling are installed for this study, and all investigators are notified of this and followed up during the research project.

8.2 Recruitment and consent

Eligible patients visiting the outpatient clinic will be asked whether they are interested to participate in this study according to standards of GCP, and if so they will receive verbal and written information. Recruitment procedures will be conducted by the investigator or the treating physician on the outpatient clinic in the different participating hospitals. Enrolment of the patients will be done according to principles stated above and the inclusion and exclusion criteria. Informed consent is required, in which the purpose of the research is explained, what the role of the subjects will be, and how the trial will be performed.

Subjects will receive the Patient Informed Form and will be able to ask any questions when they do not understand the content. They will have enough time to consider their participation in the study.
When informed consent is received the research nurse, investigator or treating physician can sign the patient informed consent. Participating in this clinical trial is voluntary and subjects can withdraw from participation for with any reason at any time.

8.3 Benefits and risks assessment, group relatedness

Participation to this study is not considered to bring any benefit to the patient. Since most of the analysis are coinciding with regular follow-up the negative impact on the subject is minimal.
Study burden for the patient is relatively small. The study procedures are stool sampling, throat swabs and blood sampling. These procedures are of minimal invasiveness.

8.4 Compensation for injury

Participation in this study is expected to be without risk because the minimal invasiveness of the study procedures. In addition, during the study there will be no (change in) medical intervention. Therefore the investigator has been granted dispensation for insurance by the METC-LDD (Medical Ethical Committee Leiden Den Haag Delft).

8.5 Role of Astra Zeneca

The company Astra Zeneca has provided the LUMC a grant for the execution of the translational research and financial support for personnel involved in the study.

9. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

9.1 Handling and storage of data and documents

The handling of personal data complies with the GDPR or General Data Protection Regulation (Dutch: Algemene Verordening Gegevensbescherming, AVG) as in effect from May 25th 2018. GDPR is an EU wide agreement which covers all member states.

All obtained material and data from patients will be handled confidentially per site. Characteristics and clinical results from participants will be collected, labelled with a measurement ID and stored in an encoded database. All data will be inserted into a database management system (eCRF) for maintenance and use of clinical data management (Castor). Each subject will be assigned a subject number at the time the informed consent is signed. Data will be encrypted**.** No characteristics that can be traced to an individual (no dates, only age) are stored or shared between the study sites.

A subject identification code list is made where the code is linked to the participant's hospital number. This list is stored securely per study site and is only accessible for local clinical investigators and members of the study team throughout the study.

Data will be accessible for the principal investigator, members of the study team, monitor and IGJ Following closure of the study.
All study records will be maintained for 15 years in a safe and secure location, as local regulations require.The investigator will retain all patient information for a period of 15 years after study completion. They will be securely stored per study site as local regulations require.

The patients can revoke their permission for participating in this study at any moment. Performed sampling and measurements will be destructed and not been used for research. If the sampling and measurements were already analysed before revoked permission they will still be used for this study.

The controller of the collected data is the principal investigator in this study. In case patients have either questions or complaints about protection of their privacy they can contact our data protection officer via privacy@lumc.nl. Information about this study, but no information about the patients, is stored at www.clinicaltrialsregister.eu

9.2 Monitoring and Quality Assurance

Monitoring in all sites in the Netherlands will be executed by (internal) monitors of the LUMC according to the monitor plan.

9.3 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.
All substantial amendments will be notified to the METC and to the competent authority.

A ‘substantial amendment’ is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

9.4 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.

9.5 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.

9.6 Public disclosure and publication policy

The study will be presented at major Lung Cancer meetings and is planned to be published in a major journal (e.g. J Thoracic Oncology, Lancet Oncology) within 6 months after full analysis of the data in agreement with the CCMO publication policy.

9.7 Synthesis

The study is considered as minimal risk and therefore a DSMB is not necessary. The monitor coordinator of the LUMC will be informed about the study procedures and will define the intensity of monitoring in this study. In the unlikely event a potential harmful finding occurs, the patient will be duly notified.

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Appendix 1

Collection of microbiome samples

Stool samples will be collected at baseline. Participants will receive a collection kit containing two sample tubes (cylindrical containers with a spatula in the lid) and gloves. They will be instructed on using these tubes. The tubes need to be stored in the refrigerator (at +/- 4 ^{0}C) in order to conserve the volatile SCFA's and calprotectin,Times of collection will be noted. These stool samples will then be collected by the investigator during the clinical visit. Stool samples can be no more than 24 hours old.
After arrival at the clinical research unit, the research personnel will immediately prepare and store one sample (meant for analysis of the gut microbiome) at -80 ^{0}C. The cooled sample will be homogenized and transferred to multiple smaller cups, which will be stored at -20 ^{0}C. The -80 ^{0}C sample will be used for 16S-rRNA metagenomic sequencing of microbial content by TNO. The -20^{0}C sample will be stored for metabolic biomarker analysis (e.g. SCFA).

Throat swabs will be collected at baseline.
Mucosal throat swab samples will be collected by trained andqualified personel. Swabs will be collected using the Copan eNAT swabs, comprising of a sterile swab and a sample tube. The sterile swab will be applied by gently stroking 5 times over the pharyngeal region. Strokes will be made in an anterior-posterior direction.
The study of throat and gut microbiota composition will be performed by (TNO, Zeist & ACTA, University of Amsterdam, Netherlands). 16S rRNA sequencing will be first performed to determine differences in bacterial phyla in responders compared with non-responders and the data will be used to study the microbiota composition in correlation with the phenotype of DCs observed by mass cytometry.

Appendix 2

CYToF analysis

T cell response against commensal bacteria: The commensal-specific T cell response will be analyzed in order to determine whether it is linked with anti-PD-L1 efficacy and/or with DC function [18] [19] . T- lymphocytes from peripheral blood and tumor lysate will be expanded in order to obtain enough cells for the analyses. An analysis of the commensal-specific T cell response will be performed in collaboration within LUMC, and functional assays will be performed as described above.

Immunophenotyping of peripheral blood from non-progressors and progressors by mass cytometry before and 4 weeks after the onset of the treatment, by using two panels (with 42 metal-labeled antibodies and 6 barcode options based on palladium metals), in order to identify circulating biomarkers as described in Exploratory Objective 2.
Using Panel 1, markers related to tumor-cell killing of $CD8^+$ T cells and NK cells will be used. Panel 2 will allow us to investigate activation and tolerogenic markers in DC subsets (e.g. pDCs, cDC1, cDC2) and monocytes.

Procedure: cells will be incubated with metal-labeled antibodies and barcoded-anti-beta2M antibodies, followed by fixation and acquisition on a Helios mass cytometer. Fcs files will be normalized using the included reference beads, concatenated and debarcoded. Then, viable immune cells will be pre-grated and exported as fcs files using Flowjo X (Treestar), followed by clustering and further analysis using the in-house developed software Cytosplore (www.cytosplore.org) and the R-package Cytofast [22] (Figure 1).

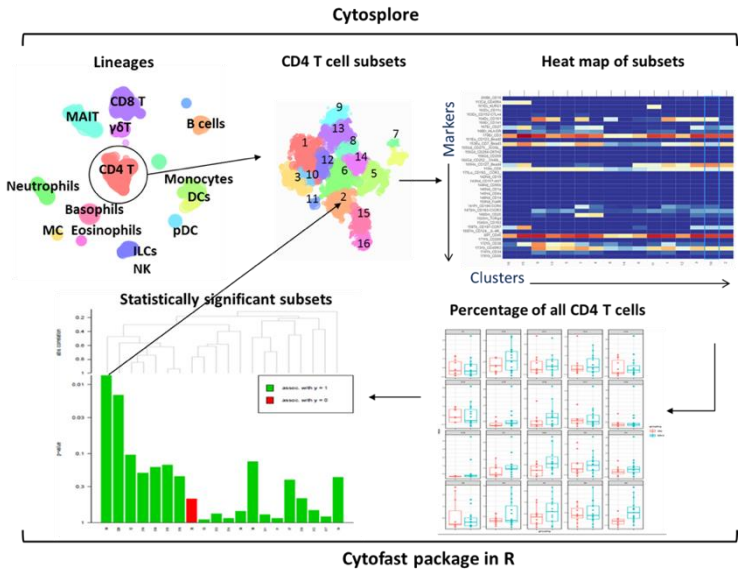

Figure 1. Pipeline for data analysis of airway tissue samples. *The fcs files generated by mass cytometry are compiled and clustered together in Cytosplore. In the first level of hierarchical clustering, the cell lineages can be identified, including T cell, B cell, NK cell, dendritic cell, monocyte and granulocyte populations. In the second level of clustering, clusters within cell lineages are identified (example here: $CD4^+$ T cells). The accompanying heatmap shows the marker expression profile of each cluster in the cell lineage (here: $CD4^+$ T cells). This is used to identify particular subsets in the cell lineage of interest. The fcs files of the clustered cells are then further analyzed in R with the Cytofast package. The abundance of each cluster per group can be represented in a quantitative bar graph. Statistical comparison is performed with 'global testing' to highlight significant changes in cluster abundance between groups.*