Advances in personalized medicine for lung cancer

Hiddinga, Birgitta

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• What's in it for the patient •

Birgitta I. Hiddinga

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What's in it for the patient

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Prof. dr. D.J. Slebos
Prof. dr. J.P. van Meerbeeck

Beoordelingscommissie

Prof. dr. H.J.M. Groen
Prof. dr. A.M.C. Dingemans
Prof. dr. V. Surmont

This thesis is a plea for finding and using biomarkers, to enhance precision medicine in the treatment of lung cancer patients, and a plea for speeding up the process towards precision medicine (or targeted therapy), because of the obvious benefits that will bring for patients and physicians, such as cutting out (or reducing) ineffective (futile ☺) therapies and medication.

Anna Boulton

Dit proefschrift is een pleidooi voor het opsporen en gebruiken van biomarkers om precisiegeneeskunde (doelgerichte therapie) te verbeteren in de behandeling van longkankerpatiënten, en een pleidooi voor de versnelde ontwikkeling hiervan, vanwege de duidelijke voordelen die dit voor patiënten met zich mee zal brengen en voor artsen, die ineffectieve (nutteloze) of overbodige therapieën en medicijnen wellicht kunnen schrappen.

CONTENTS

Chapter 1

General introduction and thesis outline

Lung cancer is a significant global health concern, accounting for a substantial proportion of cancer-related morbidity and mortality, despite all therapeutic innovations of the last decades. Understanding the epidemiology of lung cancer is essential for effective prevention, early detection, and management strategies [1]. In Europe, almost 480,000 new cases of lung cancer were diagnosed in 2020, representing almost 12% of all new cancer diagnoses, and more than 380,000 deaths related to lung cancer, corresponding to almost 20% of all cancer deaths [2]. Lung cancer remains the leading cause of cancer mortality in the Netherlands, for both males and females [3]. In the Netherlands, 15,377 new lung cancer patients were diagnosed in 2022, while 13,163 people died of lung carcinoma in 2021 [4]. In Belgium, 7,897 people were diagnosed with lung cancer in 2021 [5].

The incidence of lung cancer is strongly associated with smoking, with an estimated temporal lag of 2 to 3 decades [6]. Other risk factors include occupational exposures, such as asbestos, silica, radon, heavy metals, polycyclic aromatic hydrocarbons and air pollution by biomass. Epidemiology of lung cancer in never-smokers has revealed new insights in the etiology, such as genetic susceptibility and germ line mutations [7].
Historically and histologically, lung cancer is classified into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [8]. SCLC is highly aggressive, characterized by rapid growth and early metastasis, necessitating systemic chemotherapy as the primary treatment modality. SCLC is characterized by the (current) lack of driver mutations. NSCLC accounts for approximately 85% of all lung cancer cases and can be further subdivided into major subtypes adenocarcinoma and squamous cell carcinoma. Adenocarcinoma is the most common subtype and typically arises in the peripheral lung tissues. Squamous cell carcinoma, on the other hand, originates from the bronchial epithelium and is most often associated with a history of smoking. NSCLC has more diverse treatment options, including surgery, radiation therapy, targeted therapies and immunotherapies, depending on the specific histological subtype and stage of the disease. Moreover, recent advances in molecular profiling techniques, such as immunohistochemistry (IHC) and genetic testing, have further refined the classification of lung cancer, allowing for personalized treatment strategies based on specific biomarkers expressed by the tumor cells. Within this context, the subtyping of tumor types becomes less important, since the focus is on treatment options.

Lung cancer is a complex disease with diverse clinical outcomes and responses to treatment. Based on more than 90,000 cases of lung cancer worldwide, prognostic factors have been identified to develop the staging system of lung cancer, crucial for treatment approach and prognosis [9]. The widely accepted staging system, established by the American Joint Committee on Cancer (AJCC), employs the TNM classification. T

(tumor) represents the primary tumor size and extent, N (Nodes) indicates lymph node involvement, and M (Metastasis) reflects distant spread. Stages range from I to IV, with higher stages indicating greater tumor dissemination. Currently, the 8th edition of TNM is in use, however, edition 9 will soon become applicable [10].
Roughly, stages I to IIIC are considered treatable with curative intent, stage IV is mainly considered manageable with palliative care [11].

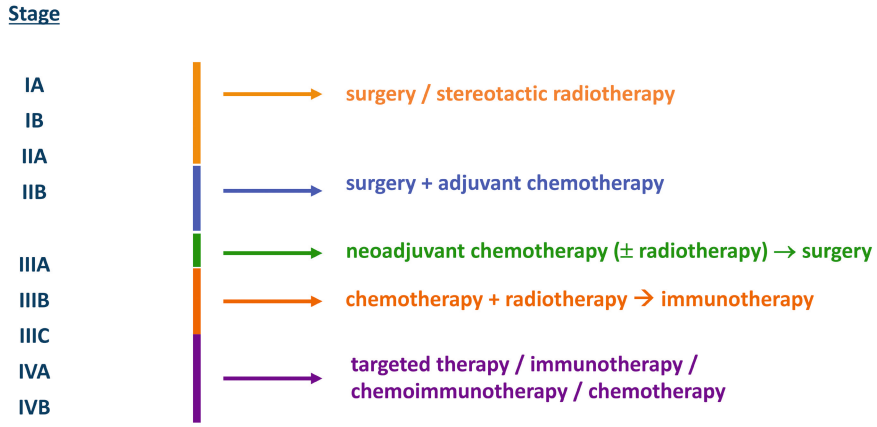

Figure 1. Algorithm therapeutic options in lung cancer

In stage I, the options of surgery or stereotactic ablative radiotherapy (SABR) are considered in a multidisciplinary meeting, with surgery leading to a 5-year overall survival rate of about 70% and about 40% after SABR. This lower survival is not explained by a lesser treatment, but is due to the composition of the patient group treated with SABR [12]. Standard treatment for stage II is surgery, potentially followed by adjuvant chemotherapy, leading to an overall 5-year survival rate of 58%, varying from 40% in elderly patients to 66% in the younger category. Alternatives for less fit patients may include SABR or conventional radiotherapy [13]. Stage III-disease is diagnosed in a heterogeneous group of patients with significant differences in tumor volume and lymph node involvement. The main intention is curative treatment, but the overall survival rates are still poor, around 30% with combined chemoradiotherapy [14]. Since the introduction of (neo-)-adjuvant immunotherapy, the 5-year survival rate increases to about 45% [15].

Treatment of NSCLC stage IV has changed since the introduction of immunotherapy with a 5-year survival rate of 6% for chemotherapy, 25% for immunotherapy and 19% for the combination, respectively [16, 17, 18]. The discovery of targeted therapy has been a major advancement for a proportion of patients with advanced NSCLC harboring an epidermal growth factor receptor (EGFR) mutation or an anaplastic lymphoma kinase

(ALK)-rearrangement [19, 20]. Patients with an EGFR mutation reach a median overall survival of 23 months, resulting in a 5-year survival rate of 11% [21]. Patients with an ALK-rearrangement have a median survival of 48 months, with a 5-year survival of 46%. Because technology is developing faster than ever before and immunotherapy is now introduced in the lower stages of the disease, the current state can be likened to a snapshot. The spectrum of various treatments and combinations of modalities may be completely different one year from now, with better outcomes.

NEUROENDOCRINE TUMORS AND NEUROENDOCRINE CARCINOMAS

Neuroendocrine tumors (NETs) and neuroendocrine carcinomas (NECs) are a distinct group, representing less than 20% of lung cancers [22]. The classification of lung NETs is primarily based on their histological features, including architectural patterns, cellular morphology, and IHC-expression. The spectrum ranges from the more benign typical carcinoid (TC) (grade 1) and atypical carcinoid (AC) (grade 2) tumors, to the grade 3 and 4 carcinomas: SCLC and large cell neuroendocrine (LCNEC), with both a high metastatic potential and a poor prognosis [23]. Their common expression, such as neuroendocrine granules and the secretion of paraneoplastic cytokines and hormones, reflects the common origin from the embryonal neuroendocrine crest. NETs arise from cells throughout the endocrine system. Although the different types of pulmonary NETs originate from the Kulchitsky cells of the bronchial mucosa, they are considered separate clinical entities that harbor different mutations causing different biology [22].

TC is characterized by well-differentiated tumor cells with uniform morphology, scarce mitotic activity, and absence of necrosis. AC, on the other hand, exhibits more aggressive features, including increased mitotic activity and focal necrosis [23]. LCNEC is characterized by sheets or nests of large cells with abundant cytoplasm, vesicular nuclei and prominent nucleoli.

IHC plays a vital role in the diagnosis and subclassification of lung NETs and NECs [24]. IHC markers, such as chromogranin A and synaptophysin, are used in confirming the neuroendocrine nature of the tumor, whereas CD56 and TTF-1 are more commonly expressed in NECs. LCNEC represents an intermediate grade neuroendocrine malignancy with both neuroendocrine and non-neuroendocrine components. Histologically, IHC markers such as synaptophysin, chromogranin A and CD56, are expressed in LCNEC, while TTF-1 expression may vary. Whereas Ki-67 has proved its role as a diagnostic and prognostic factor in gastro-entero-pancreatic NETs, the value in lung NETs is still debated [25].

SCLC is the most aggressive subtype, associated with a high proliferation rate and rapid metastasis. Histologically, SCLC is characterized by small cells with scant cytoplasm, finely granular chromatin, and high mitotic activity. The classic morphological features of SCLC include nuclear molding, extensive necrosis, and a high nuclear-to-cytoplasmic ratio. SCLC demonstrates strong positivity for neuroendocrine markers such as synaptophysin, chromogranin A and CD56, with variable expression of TTF1.

IHC helps to distinguish between different subtypes and contributes to personalized treatment decisions.

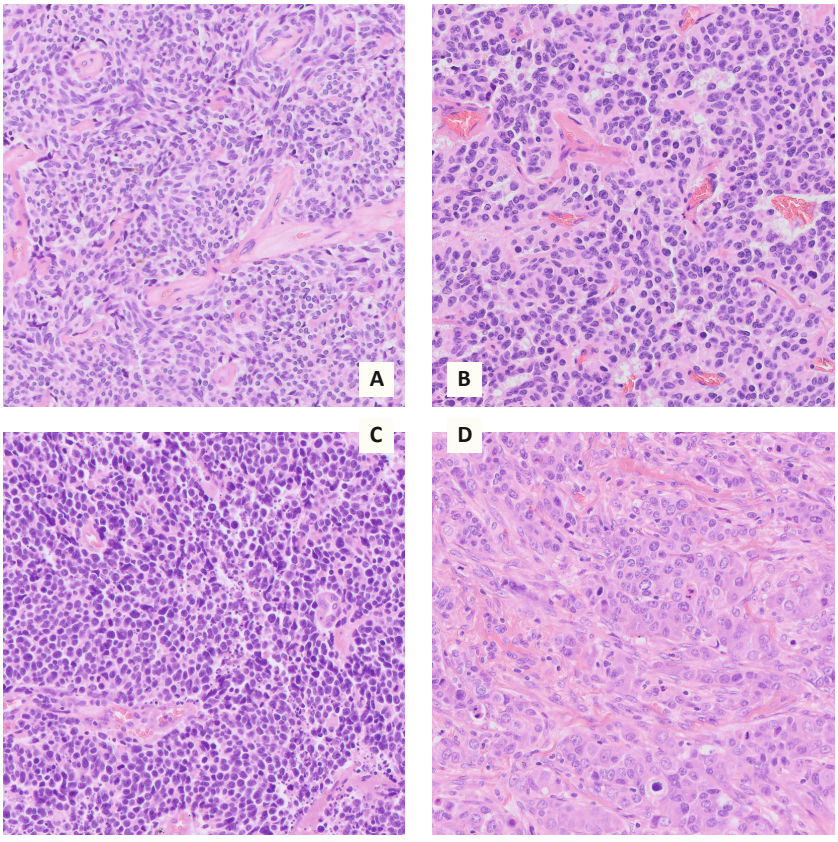

Figure 2. Characteristic microscopic images of the 4 main types of neuroendocrine tumors in the lung (all haematoxylin and eosin stained): A. Typical carcinoid, B. Atypical carcinoid with central mitosis, C. Small cell lung carcinoma, D. Large cell neuroendocrine carcinoma.

BIOMARKERS

Historically, biomarkers in lung cancer were based on clinical and pathological features in the patient that are associated with disease progression and overall survival, independent of therapy, so-called prognostic factors [26]. Prognostic biomarkers provide information about the natural course of the disease, while predictive biomarkers help identify patients who are more likely to respond to specific treatments [27]. For instance, the presence of EGFR mutations or ALK rearrangements in NSCLC has been identified as a strong prognostic indicator in NSCLC. EGFR mutations or ALK rearrangements predict the response to targeted therapies, such as EGFR tyrosine kinase inhibitors (TKIs) or ALK inhibitors (ALKi), respectively. Similarly, the expression of programmed death-ligand 1 (PD-L1) has emerged as a predictive biomarker for the response to immune checkpoint inhibitors in NSCLC. The development of immunotherapy offers new perspectives in the treatment of NSCLC for a subset of patients, but lung cancer remains a fatal condition nevertheless [16].

Prognostic and predictive biomarkers have emerged as valuable tools in guiding treatment decisions and predicting patient outcomes. Their integration into clinical practice allows for personalized treatment strategies, optimizing therapeutic efficacy while minimizing unnecessary toxicities. Moreover, ongoing research aims to identify novel biomarkers and therapeutic targets to further enhance patient outcomes and improve treatment selection.

In the first part of this thesis, we investigate whether rare tumors such as NETs and NECs harbor biomarkers that were found in other tumor types to enable repurposing of drugs so patients can benefit from drugs that are already approved for other tumor types. Key questions are: What do we test and what medication is available as treatment of lung cancer? Can biomarkers act as a target for precision therapy and can biomarkers help us to evaluate therapy in order to detect early progression in a non-invasive manner? Will we be able to identify patients with biomarkers to stratify them for specific treatment options?

FINDING TARGETS FOR REPURPOSING OF DRUGS

SCLC is highly aggressive and frequently metastasizes at the time of diagnosis. It is characterized by an early response to chemotherapy but has a high rate of relapse. For patients with relapsed or refractory SCLC, the only approved second line therapy is topotecan [28]. For patients with NETs and NECs, treatment options remain limited,

despite the fact that the therapeutic arsenal has grown considerably [29]. Most recent attempts to allow new therapies to find their way into the clinic have failed.
It is ethical to include patients with relapsed or refractory SCLC without druggable driver mutations in clinical trials. Repurposing of drugs used for other indications and /or tumor types is an acceptable and innovative strategy to improve prognosis for patients with SCLC [30]. For example, in patients with glioblastoma multiforme and refractory astrocytoma whose tumor contained a methylated MGMT promoter, a survival benefit was observed when they were treated with radiotherapy and temozolomide [31]. Temozolomide is an alkylating agent that facilitates epigenetic gene silencing of the *MGMT* gene by promoter methylation. This is associated with the loss of MGMT expression, triggering cytotoxicity and apoptosis. Temozolomide is approved for use in patients with glioblastoma multiforme and in refractory astrocytoma and is usually well tolerated [32]. SCLC has aberrantly methylated methylguanine DNA methyltransferase (MGMT) [33]. In a retrospective series, the presence of the methylated *MGMT* promotor gene was 48% [34]. No prospective data are available in SCLC. Temozolomide has shown beneficial effects in patients with relapsed SCLC, especially in a subgroup with MGMT promoter methylation [34]. Stratification to MGMT promoter hypermethylation status might help select an SCLC patient population that can benefit from treatment with temozolomide.

In rare tumors with high medical need, it is important to overcome cancer-specific properties and to aim for targets that can influence outcome of therapy [30]. These targets are not only important as a guide for treatment options, but also to expand the range of therapeutic options. Furthermore, unravelling the genome of SCLC and discovering biomarkers is crucial for making choices in treatment [35].

K-RAS MUTATION IN NSCLC

The Kirsten rat sarcoma viral oncogene homologue (*K-ras*) mutation is the most frequent genetic alteration found in NSCLC [36]. *K-ras* mutations occur in approximately 20-30% of NSCLC cases and are associated with heterogeneity in clinical characteristics and a poor prognosis to standard NSCLC therapies [37, 38]. The *K-ras* gene plays a crucial role in cell signaling pathways regulating cell growth and survival [39, 40]. Mutations in *K-ras* result in constitutive activation of downstream signaling pathways, promoting uncontrolled cell proliferation. In NSCLC, a paradigm shift occurred with the introduction of targeted therapy and immunotherapy. Limited data is available in *K-ras* mutated NSCLC and patients never really benefited from these improvements, until recently. With new targeted drugs on the horizon for a subgroup of the *K-ras* mutant NSCLC, immunotherapy can be considered the mainstay in the treatment of most *K-ras* mutated NSCLC patients

[41, 42]. Ongoing research focuses on developing novel therapies targeting *K-ras* mutant lung cancer, aiming to improve outcomes for this subgroup of patients.
In the second part of the thesis, we report on the feasibility studies in a cohort of patients with advanced *K-ras* mutated NSCLC. In the early days of treatment with immunotherapy in NSCLC, PD-L1 was used as a biomarker to predict responses to therapy [43]. However, when we started this study in 2015, we did not use PD-L1 to make therapy decisions, although *K-ras* mutation was mandated to enter the study once the other inclusion criteria were fulfilled.

CIRCULATING TUMOR DNA AND GUT MICROBIOME

In addition to the radiology assessment of response to treatment, patient 'materials', such as circulating tumor DNA (ctDNA) in the blood and the defense mechanism in the gut (gut microbiome), have attracted considerable attention in recent years [44]. As the gut microbiome is associated with response to immunotherapy, investigation is needed to verify whether the gut microbiome and circulating tumor DNA can act as selection criteria to treat patients with immunotherapy. Furthermore, patients would benefit if gut microbiome in combination with circulating tumor DNA in the blood could be used to simplify investigations. It would make it possible to closely monitor response and, should treatment fail, detect progressing disease more quickly, to prevent the unnecessary continuation of unhelpful medication. In this thesis, we investigated ctDNA and gut microbiome in a selected group of NSCLC patients harboring a *K-ras* mutation. During this time, immunotherapy became available as second or further line therapy after chemotherapy. The role of PD-L1 was not clear yet, and patients qualified for treatment with nivolumab regardless of PDL1-status.

TISSUE

Innovations are moving fast. Molecular profiling techniques, such as next generation sequencing have become increasingly important in routine cancer diagnostics. Now that we are better able to examine the tumor's characteristics and are gaining more insight into treatment options, we need better biomarkers to predict the outcome of the chosen therapy [39, 40]. New technologies, such as exhaled breath markers and metabolomics, are entering the field. Integration with next generation sequencing, imaging, radiomics and artificial intelligence is our future [45]. Meanwhile, obtaining tissue remains of the utmost importance, as tumor tissue is the "gold standard" to detect genetic alterations. Diagnostic techniques for assessing pulmonary lesions remain challenging. Transthoracic biopsies are currently the gold standard, and, although accurate, they are associated with an increased risk of complications [46]. With newer bronchoscopy techniques, such

as virtual bronchoscopy navigation (VBN), it is possible to obtain tissue from difficult-to-reach abnormalities in the lung and to carry out next generation genetic sequencing to offer the appropriate treatment [47]. Or, in case of progressive disease, these techniques also make it possible to obtain new tissue and assess resistance mechanisms for a different choice of therapy. An adequate supply of tissue is important for the purpose of molecular analysis within the context of moving towards precision medicine.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is

- to investigate the role of certain biomarkers in neuroendocrine tumors and neuroendocrine carcinomas
- to prospectively estimate the diagnostic relevance of ctDNA and gut microbiome in patients with non-small cell lung cancer
- to prospectively evaluate the role of virtual bronchoscopy navigation in patients with lung nodules, to obtain tissue for diagnosis and molecular analysis in order to optimize treatment.

Chapter 2 provides an overview of recent progress in the field of the systemic treatment of SCLC.

Chapter 3 contains an editorial written for a special issue on “Targeted therapy for small cell lung cancer”, revealing potential biomarkers to target therapy and to enable stratification of patient groups for more effective treatments.

As immunotherapy has moved forward to the lower stages of SCLC and new biomarkers have been revealed since the publication of chapters 2 and 3, we have added an **Addendum** (Current developments in the treatment of SCLC) to these chapters with information about the latest developments in the field of SCLC and specifically how targets can be found in different tumor types such as NETs and LCNECs to expand therapeutic options.

Chapter 4 deals with a systematic review of O6-Methylguanine-DNA methyltransferase (MGMT) in lung cancer conducted to evaluate whether MGMT promoter methylation can act as a prognostic or predictive biomarker to help select patients with lung cancer who may benefit from therapy with temozolomide.

Although novel targets in NETs and NECs are needed to improve outcome, our review shows that the presence of MGMT promoter methylation in NETs and NECs may act as a

predictive marker for response on treatment with temozolomide [34, 48]. The hypothesis is included that ALK rearrangement may act as a biomarker in patients with NETs and NECs, since ALK plays an important role in the nervous system [49].

In **chapter 5** we focus on a retrospective analysis performed on tissue samples of patients with NETs and NECs, taken to establish the frequency of MGMT promoter methylation and the frequency of ALK expression and rearrangement.

In **Chapters 6 and 7**, the relevance of monitoring ctDNA in blood samples is investigated as well as the gut microbiome in a Dutch cohort of patients treated with anti-PD-1 immunotherapy for advanced *K-ras* mutated NSCLC.

In **chapter 8** we present a 'Lesson of the Month', revolving around an elderly patient with chronic myelomonocytic leukemia who was diagnosed with lung adenocarcinoma. Genetic testing of the lung tumor revealed several mutations, indicating a second primary malignancy that would not have been found by histopathology alone, stressing the importance of molecular analysis of tumor tissue.

Chapter 9 comprises a description of a single-center, prospective, observational study - NAVIGATOR - of patients undergoing a virtual bronchoscopy navigation (VBN) procedure to assess a pulmonary nodule. The correct diagnosis of a pulmonary nodule will allow for a more effective treatment plan, preventing unnecessary or more invasive procedures, such as surgery. We report on the diagnostic yield and the adaptation of treatment plans based on tissue and molecular analysis.

In **chapter 10** we highlight the outcomes of the thesis and put them in perspective, with a glance at the future.

Hoofdstuk 11 is de Nederlandse samenvatting waarin we de uitkomsten van dit proefschrift in perspectief plaatsen en een blik werpen op de toekomst.

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Chapter 2

Recent developments in the treatment of small cell lung cancer

Birgitta I. Hiddinga
Jo Raskin
Annelies Janssens
Patrick Pauwels
Jan P. van Meerbeeck

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ABSTRACT

Small cell lung cancer (SCLC) comprises about 15% of all lung cancers. It is an aggressive disease, with early metastasis and a poor prognosis. Until recently, SCLC treatment remained relatively unchanged, with chemotherapy remaining the cornerstone of treatment. In this overview we will highlight the recent advances in the field of staging, surgery, radiotherapy and systemic treatment. Nevertheless, the prognosis remains dismal and there is a pressing need for new treatment options. We describe the progress that has been made in systemic treatment by repurposing existing drugs and the addition of targeted treatment. In recent years, immunotherapy entered the clinic with high expectations of its role in the treatment of SCLC. Unravelling of the genomic sequence revealed new possible targets that may act as biomarkers in future treatment of patients with SCLC. Hopefully, in the near future, we will be able to identify patients who may benefit from targeted therapy or immunotherapy to improve prognoses.

Tweetable Abstract

Advances in radiotherapy, targeted treatment and immunotherapy are limited. Progress in treatment options are needed for the treatment of SCLC. Exome sequencing to identify targetable biomarkers could select patients who would benefit from certain therapies.

INTRODUCTION

Small cell lung cancer (SCLC) is an extremely aggressive tumor type which accounts for about 15% of lung cancer cases [1, 2]. The cancer originates from neuroendocrine precursor cells and is characterized by its rapid growth and early metastasis, with more than 70% of patients presenting with metastasized disease [3]. Approximately 10–25% of patients have brain metastases at initial diagnosis, and an additional 40–50% will develop them during the course of their disease [3]. First-line treatment in metastatic SCLC consists of a combination of platinum and etoposide [4]. However, the majority of patients experience relapse within the first year of treatment, resulting in poor survival. Several agents or addition of a third drug have failed to show any improvement in outcomes. Even for patients without metastases at diagnosis, the curation rate remains low. Therefore, there is a high unmet need for therapies that could improve survival in patients with SCLC. The guidelines of the European Society of Medical Oncology (ESMO) and the American College of Chest Physicians (ACCP) endorsed by the American Society of Clinical Oncology (ASCO) have not been updated since 2013 [1, 5]. In this manuscript we will address the recent progress achieved in the field of staging, surgery, radiotherapy and systemic treatment, such as immunotherapy, since the landmark reviews published in the beginning of last decade [6, 7].

EIGHTH TUMOR-NODE-METASTASIS CLASSIFICATION

The prognosis of SCLC depends on the tumor stage. Previously, the classifications limited disease (LD-SCLC) and extensive disease (ED-SCLC) were used, where limited disease was defined as disease confined to the ipsilateral hemithorax, which can safely be encompassed within a single radiation field [8]. The Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) staging system was developed for non-small cell lung cancer (NSCLC), and edition 8 uses tumor size and the number of metastases and affected organs to estimate prognoses for the different stages of this disease [9]. In clinical practice, as well as in clinical trials, the distinction between LD-SCLC and ED-SCLC is still useful when deciding on a treatment plan.

SURGERY IN VERY LIMITED SCLC

Surgery in SCLC is not widely accepted but can be considered for very small biopsy-proven tumors (very limited disease), cT1N0M0, with confirmed negative mediastinal staging. Most commonly, a surgically removed lung nodule of unknown origin turns out to be a small SCLC. In a systematic review, surgery was not supported in limited SCLC [10]. In several series data about correct staging and adjuvant therapy was unclear. Invasive

mediastinal staging is mandatory. There is a tendency towards offering surgery for very small SCLC with negative lymph nodes, but concurrent chemoradiation is an alternative choice. Data about adjuvant radiotherapy and adjuvant chemotherapy are insufficient to offer strong recommendations [1]. Prospective studies are needed to define the role of surgery and adjuvant treatment of very small SCLC.

RADIOTHERAPY IN LD-SCLC

For LD-SCLC, which represents around 30% of newly diagnosed SCLC, the standard treatment with curative intent consists of four cycles of platinum-doublet chemotherapy combined with radiotherapy, which improves overall survival compared with chemotherapy alone, even in elderly patients [11]. A concurrent approach is preferred, based on a median survival time of 27.2 months in the concurrent arm, compared with 19.7 months in the sequential arm of a Japanese trial [12]. Timing of radiotherapy is crucial, the shorter the overall treatment time, the better the 5-year overall survival (OS), with the start of radiotherapy preferably coinciding with the first or second cycle of chemotherapy [13, 14]. Despite the older phase 3 trials preferring twice-daily radiotherapy, once-daily radiotherapy is still the standard of care in most centers for practical reasons, and most patients do not qualify for twice-daily radiotherapy due to comorbidity and performance status [15].

The phase 3 trial CONVERT compared once-daily radiotherapy (66 Gy in 6.5 weeks) with twice-daily radiotherapy (45 Gy in 30 fractions in 3 weeks) concurrently with platinum/etoposide chemotherapy in localized SCLC [16]. The study was designed as a superiority trial, with the comparison of OS in both arms as the primary end-point, hoping to demonstrate the benefit of twice-daily irradiation. Of the 547 patients randomized, the two-year OS was 56% in the twice-daily radiotherapy arm versus 51% in the conventional arm, which was not statistically significant. No difference in toxicity between both arms was reported.

Encouraged by the assumed noninferiority and safety of twice-daily radiotherapy in SCLC, a Scandinavian phase 2 trial compared the efficacy and tolerability of standard-dose 45 Gy in 30 fractions twice-daily and a high-dose 60 Gy in 40 fractions twice-daily, hoping to improve local control and thus survival [17]. Patients receiving the higher dose had a significantly longer 2-year OS (73% versus 46%; p=0.002) and median OS (42 months versus 23 months; p=0.027), without – unexpectedly – an increase in toxicity, and comparable tolerance for both arms [18]. The conclusion is that higher dose radiotherapy twice-daily in limited disease SCLC is feasible and tolerable compared with 45 Gy. The currently ongoing CALGB 30610 phase 3 trial is comparing once-daily high-dose thoracic

radiotherapy (70 Gy/45 fractions) with standard twice-daily radiotherapy (45 Gy) [19]. The other experimental arm (61.2 Gy) was discontinued at interim analysis.

These trials may shed more light on the issue of the optimal radiation schedule for radiotherapy in locally advanced SCLC.

RADIOTHERAPY IN ED-SCLC

Prophylactic cranial irradiation

As the brain is a common site of distant failure in patients with SCLC, prophylactic cranial irradiation (PCI) is recommended in patients after curative treatment for limited stage disease [20]. In an older meta-analysis, the incidence of brain metastases decreased more than 25% 3 years after PCI, with a doubling of survival, 42% versus 23% at 2 years [21]. However, these trials predate the current, more sensitive staging with MRI and positron emission tomography (PET) scans.

In a prospective trial, patients with stage IV SCLC with any response to chemotherapy, were randomized to PCI or no further treatment with the time to symptomatic brain metastases as the primary end-point [3]. Patients in the irradiated group had a lower risk of brain metastases at 1 year: the cumulative risk was 14.6% in the irradiated group and 40.4% in controls. The 1-year survival rate was 27.1% in the irradiation group and 13.3% in controls. PCI appeared to be an effective add-on therapy, although the optimal total dose and fractionation schedule remains uncertain. Furthermore, the absence of systematic brain imaging before entering the study did raise concerns about the findings of this study. A Japanese phase 3 trial reassessed the efficacy of PCI in patients with metastasized SCLC with any response to chemotherapy [22]. Patients without brain metastases on MRI were randomized to PCI (25 Gy in 10 daily fractions of 2.5 Gy) or observation. After a planned interim analysis, the study was closed due to futility. The likelihood that PCI would be superior to observation at the end of the study was minimal. At 12 months follow-up, PCI reduced the incidence of brain metastases (32.9% versus 59%) but did not improve OS (48% versus 54% at 1 year).

The results of the Japanese PCI study challenge the benefits of PCI. Although PCI is generally well tolerated, patients experience fatigue, nausea, cognitive decline and ataxia [23]. These adverse events may be mild and transient, but could also be progressive and persistent with structural brain damage on MRI. Currently, the guidelines support PCI if patients respond to chemotherapy. The accepted radiation dose is 25 Gy in 10 fractions of 2.5 Gy. There is no role for routine hippocampal sparing [24]. The results of the Japanese trial has led to the dismissal of PCI in many centers. The EORTC Lung Cancer

Group is developing a randomized study of PCI *versus* watchful waiting with periodic brain MRI (PRIMALung). A randomized phase III study of the South-West Oncology Group is randomizing patients to either MRI surveillance alone, or MRI surveillance with PCI [25].

Consolidation thoracic radiotherapy

Intrathoracic tumor control after chemotherapy remains a problem, as most patients have persistent disease, with disease progression within 1 year. Beneficial effects of thoracic radiotherapy were described in a retrospective series [26]. In a randomized phase 3, after completion of chemotherapy and PCI, thoracic irradiation (30 Gy in 10 fractions) was performed, resulting in an OS at 2 years of 13% versus 3% in controls [27]. The authors conclude that thoracic radiotherapy should be considered for patients with advanced disease with any response to chemotherapy. However, in clinical practice this advice is not implemented. Potentially, only patients with presenting symptoms of vena cava superior syndrome, central airway compression, or atelectasis of the lung may benefit from consolidation thoracic radiotherapy [27].

FIRST-LINE SYSTEMIC TREATMENT IN METASTASIZED SCLC

SCLC is very sensitive to chemotherapy and treatment usually induces rapid responses. The current first-line treatment in ED-SCLC is platinum-based chemotherapy, four to six cycles of cis- or carboplatin plus etoposide in Europe and the United States, and platinum plus irinotecan in Japan [4]. Carboplatin is generally preferred over cisplatin due to its similar efficacy and lower toxicity [28]. However, the majority of patients experience a relapse within the first year of treatment: some of them during treatment (platinum-resistant), some within 90 days from the treatment interruption (platinum-refractory) and others 90 days or more after treatment stop (platinum-sensitive) [29]. In platinum-sensitive relapse, rechallenge with first-line chemotherapy is preferred [1, 5]. Adding a third cytostatic agent to this therapeutic backbone has previously been shown not to result in a better outcome [6].

SECOND-LINE THERAPY IN SCLC

Topotecan is the only drug that is formally approved as second-line treatment for SCLC and remains the standard of care. Oral topotecan had a response rate (RR) of 6–17% and a median survival of 25.9 weeks compared with a median survival of 13.9 weeks in a group that was assigned to best supportive care [30].

IMMUNOTHERAPY

There is great interest in whether immune checkpoint inhibition (ICI) might play a role in the treatment of SCLC. The rationale for combining immunotherapy with chemotherapy in SCLC is the high mutational burden in this tumor, with potentially enhanced immunogenicity. Chemotherapy may stimulate the expression of tumoral antigens, priming the tumor for response to checkpoint inhibitory therapy.

Immunotherapy in adjuvant setting after curative chemoradiotherapy

Despite good initial responses to definitive treatment with curative chemoradiotherapy, outcomes remain poor, with a median progression free survival (PFS) of 15 months and OS of 25 months. The role of adjuvant immunotherapy in this setting was explored (table 1).

Table 1. Immunotherapy trials in small-cell lung cancer (SCLC)

Study [ref]	Trial design	Medication	Number of patients	PFS (in mo)	OS (in mo)
		Adjuvant in limited disease			
STIMULI [31]	Phase 2, open-label	1: nivolumab + ipilimumab 2: observation	153, closed early	10.7	was not met
ADRIATIC [32]	Phase 3, RCT, double-blind	1: durvalumab + placebo 2: durvalumab + tremelimumab 3: placebo + placebo	600, recruiting		
		First line in metastasized SCLC			
NCT01331525 [33]	Phase 2, RCT, double-blind	1: carboplatin / etoposide + ipilimumab 2: carboplatin / etoposide + placebo	42	6.9 1 year-PFS: 15.8% (6 of 35 pts)	17.0

Table 1. Continued

Study [ref]	Trial design	Medication	Number of patients	PFS (in mo)	OS (in mo)
NCT00527735 [34]	Phase 2, RCT, double-blind	1: carboplatin / paclitaxel + placebo (control arm)	130	5.2	12.9
		2: carboplatin / paclitaxel + ipilimumab followed by paclitaxel + carboplatin + placebo (concurrent arm)		3.9	9.1
		3: carboplatin / paclitaxel + placebo followed by carboplatin / paclitaxel + ipilimumab (phased arm)		5.2	9.9
IDEATE [35]	Phase 3, RCT, double-blind	1: cisplatin / etoposide + ipilimumab	1,132	4.6	11.0
		2: cisplatin / etoposide + placebo		4.4	10.9
IMpower133 [36, 37]	phase 3, RCT, double-blind	1: carboplatin / etoposide + atezolizumab	403	5.2	12.3
		2: carboplatin / etoposide + placebo		4.3 HR PFS 0.78	10.3 HR OS 0.73
CASPIAN [39, 40]	Phase 3, RCT, open-label	1: platinum / etoposide + durvalumab + tremelimumab	268	4.9 HR PFS 0.84	10.4 HR OS 0.82
		2: platinum / etoposide + durvalumab	268	5.1 HR PFS 0.78	12.9 HR OS 0.73
		3: platinum / etoposide	269		10.5

Study [ref]	Trial design	Medication	Number of patients	PFS (in mo)	OS (in mo)
KEYNOTE-604 [42]	Phase 3, RCT, double-blind	1: platinum / etoposide + pembrolizumab	453	4.5	10.8
		2: platinum / etoposide + placebo		4.3 HR PFS 0.75	9.7 HR OS 0.80
ECOG-ACRIN EA5161 [45]	Phase 2, RCT, double-blind	1: platinum / etoposide + nivolumab	160	5.5	11.3
		2: platinum / etoposide		4.6 HR PFS 0.68	11.3 HR OS 0.67
REACTION [46]	Phase 2, RCT,	1: platinum / etoposide + pembrolizumab		5.4	12.3
		2: platinum / etoposide + placebo		4.7 HR PFS 0.84	10.4 HR OS 0.73
Maintenance after first-line chemotherapy					
NCT02359019 [47]	Phase 2, single arm	pembrolizumab	45	1.4 irPFS 4.7	9.2
Checkmate 451 [48]	Phase 3, RCT, double-blind	1: nivolumab	280		
		2: nivolumab + ipilimumab	279	HR PFS 0.67	HR OS 0.84
		3: placebo	275	HR PFS 0.72	HR OS 0.92
RAPTOR [49]	Phase 2 -3, RCT	1: atezolizumab after response on chemotherapy and atezolizumab	138 phase 2 186 phase 3	Primary endpoint PFS in phase 2	
		2. atezolizumab + (extra-)thoracic radiotherapy after response on chemotherapy and atezolizumab		Primary endpoint OS in phase 3	
Progression after first-line chemotherapy					
Checkmate 331 [50]	Phase 3, RCT	1: nivolumab 2: topotecan	569		7.5 8.4

Table 1. Continued

Study [ref]	Trial design	Medication	Number of patients	PFS (in mo)	OS (in mo)
Checkmate 032 [51]	Phase 1 / 2, open-label	Nivolumab +/- ipilimumab in different dosages	216		
		1: nivolumab 3 mg/kg			1: ORR 10%
		2: nivolumab 1 mg/kg + ipilimumab 3 mg/kg			2: ORR 23%
		3: nivolumab 3 mg/kg + ipilimumab 1 mg/kg			3: ORR 19%
Checkmate 032 [52]	Phase 1 / 2, open-label	Nivolumab monotherapy 3 mg/kg beyond 3rd line			ORR 11.9%
KEYNOTE-028 [54]	Phase 1b, single arm	Pembrolizumab	24		9.7 ORR 33%
KEYNOTE-158 [55]	Phase 2, single arm	Pembrolizumab	107	2.0	9.0 (PDL1+ 14.6 mo and in PDL1- 7.7 mo) ORR 18.7%, ORR in PDL1+ 35.7% and in PDL1-:6.0%
IFCT-1603 [56]	Phase 2, randomized, 2:1	1: atezolizumab	49	1.4	9.5
		2: conventional chemotherapy	24	4.3	8.7
BALTIC [57]	Phase 2, open-label	A:durvalumab + tremelimumab	21		ORR 9.5%
MISP-MK3475 [58]	Phase 2, single-arm, open-label	Paclitaxel + pembrolizumab	26	5.0	9.1 ORR 23.1%

SCLC: small-cell lung cancer; RCT: randomized controlled trial; HR: hazard ratio; OS: overall survival; PFS: progression free survival; irPFS: immune related progression free survival; ORR: objective response rate; PD-L1: programmed death ligand-1.

The STIMULI phase 2 study of maintenance nivolumab plus ipilimumab in LD-SCLC was conducted to evaluate whether adjuvant immunotherapy might improve outcomes after completion of concomitant chemoradiotherapy and PCI [31]. After randomization of 153 patients of the 222 planned patients, the study was closed due to slow accrual. It did not meet its primary end-point of improving PFS (10.7 months *versus* 14.5 months). Treatment failure in the immunotherapy arm was mostly due to toxicity; treatment failure in the observation arm was due to disease progression.

In the ongoing phase 3 ADRIATIC trial, 600 patients with at least stable disease after concomitant chemoradiotherapy, with or without PCI, will be randomized 1:1:1 to receive durvalumab plus placebo, durvalumab plus tremelimumab, or double placebo for a maximum of 24 months [32]. Primary end-points are PFS and OS for durvalumab, with or without tremelimumab, compared with placebo. Currently, adjuvant ICI have no role in the treatment of locally advanced SCLC after completion of chemoradiotherapy with or without PCI.

Immunotherapy in first-line metastasized SCLC

Two pivotal phase 2 studies introduced immunotherapy to SCLC, combining the CTLA-4 inhibitor ipilimumab with first-line chemotherapy [33, 34] (table 1). In the first study, the primary end-point of 1-year PFS was not met [33]. The second phase 2 study showed a slightly better outcome for patients treated in the phased ipilimumab versus concurrent ipilimumab with chemotherapy [34].

In the large phase 3 study, IDEATE, patients were randomly assigned to receive chemotherapy (platinum/etoposide) plus ipilimumab or placebo every 3 weeks for a total of four doses in a phased schedule [35]. The primary end-point OS was not met, with higher toxicity in the chemotherapy/ipilimumab arm. Ipilimumab may not be effective without corresponding T-cell activation in the tumor environment.

The IMPOWER-133 study was designed to evaluate the safety and efficacy of atezolizumab versus placebo in combination with carboplatin/etoposide in 403 treatment-naive participants with metastasized SCLC [36]. The hazard ratio (HR) for disease progression or death was 0.77 (p=0.02). The addition of atezolizumab to chemotherapy in the first-line treatment of metastasized SCLC resulted in a longer OS (33.5% long-term survivors versus 20.4% for placebo) and PFS than chemotherapy alone [37]. Although only 43% of tumor specimens were evaluable for programmed death ligand-1 (PD-L1), neither PD-L1 nor the tumor mutational burden (TMB) were found to discriminate long-term survivors. Chemotherapy plus atezolizumab had a comparable safety profile to chemotherapy alone, and did not result in impaired quality of life [38]. Atezolizumab has been approved

for registration by both the US Federal Drugs Administration (FDA) and the European Medicines Agency (EMA) [2].

In the phase 3 CASPIAN-trial, the addition of durvalumab and tremelimumab to chemotherapy was also evaluated in treatment-naive patients with metastasized SCLC [39]. Patients were randomly assigned (in a 1:1:1 ratio) to durvalumab plus chemotherapy, durvalumab/tremelimumab plus chemotherapy, or platinum/ etoposide alone. First-line durvalumab plus chemotherapy significantly improved OS (22% after 24 months) in patients with advanced SCLC compared with chemotherapy alone. No additional benefit of tremelimumab was observed [40]. However, three times more patients derived long-term benefit when treated with durvalumab plus chemotherapy compared with chemotherapy alone [41]. Patients in all arms with a PFS >12 months had improved overall response rate (ORR), duration of response and OS compared with the PFS pembrolizumab arm, the significance threshold was not met (HR 0.8, 95% CI 0.61–0.98). ORR was 71% in the pembrolizumab arm and 62% for placebo. Adding pembrolizumab to chemotherapy did not decrease quality of life [43, 44].

These phase 3 studies showed an improved OS and PFS by adding immunotherapy to first-line chemotherapy, with an acceptable safety profile and quality of life, which supports this regimen as standard of care.

The phase 2 ECOG-ACRIN EA5161-study randomized between platinum/etoposide plus maintenance nivolumab and platinum/etoposide plus observation [45]. The median PFS (primary end-point) and OS were clinically significant, 5.5 months and 11.3 months in the nivolumab plus chemotherapy arm versus 4.6 months and 9.3 months in the chemotherapy arm, respectively.

In the phase 2 REACTION-study, chemotherapy with or without pembrolizumab in first-line treatment showed similar results with a not significant PFS of 5.4 months versus 4.7 months [46].

Maintenance immunotherapy after first-line chemotherapy

The results of maintenance immunotherapy after completion of first-line chemotherapy are disappointing [47, 48]. In the phase 3 CheckMate-451 trial, patients with responses after completion of first-line chemotherapy were randomized between nivolumab, nivolumab with ipilimumab, or placebo as maintenance therapy [48]. Maintenance immunotherapy did not improve OS, but favorable PFS suggests that some patients could have benefited from maintenance therapy. In a phase 2 study with pembrolizumab, PD-L1 could be assessed in 30 of 45 patients and was positive (PD-L1 expression >1%)

in three patients [47], having a PFS of 10, 11 and 13 months. Each unit increase in baseline circulating tumor cells correlated with worse PFS (p=0.052; adjusted for brain metastases, age and gender). Biomarkers to identify the patients most likely to benefit from immunotherapy in the maintenance setting are also lacking.

The RAPTOR trial is evaluating a new strategy of whether thoracic radiotherapy plus maintenance atezolizumab after response on first-line atezolizumab and chemotherapy is better than atezolizumab maintenance alone in patients with metastasized SCLC [49].

Immunotherapy in second-line therapy and beyond

Nivolumab has been investigated in the phase 3 CheckMate-331 trial versus topotecan or amrubicin as second-line therapy after progression on standard chemotherapy [50]. The study resulted in a median OS of 7.5 months with nivolumab versus 8.4 months with chemotherapy (HR 0.86, 95% CI 0.72–1.04). Immunotherapy in second-line therapy showed no improvement in therapy for SCLC.

Ongoing approaches include combinations of anti-CTLA-4 (ipilimumab and tremelimumab) and anti-PD-(L)1 therapy (nivolumab, pembrolizumab, atezolizumab and durvalumab). In the CheckMate-032 trial, nivolumab/ ipilimumab, and nivolumab alone, were evaluated in pretreated patients with SCLC [51, 52]. The responses were fast and durable for patients with relapsed SCLC, regardless of platinum sensitivity or PD-L1 status. Nivolumab monotherapy is approved in the United States as third-line or later based on the pooled data of this trial. In a separate analysis of the pooled nivolumab monotherapy cohort in CheckMate-032, the ORR was 21.3% in patients with a high TMB versus 4.8% in those with a low TMB [53]. In the nivolumab plus ipilimumab arm the efficacy was also enhanced in the high TMB group, suggesting TMB may nevertheless have a role as a biomarker for immunotherapy in SCLC, but this has to be further explored.

Several phase 1 and 2 studies evaluated diverse ICIs, which all failed to show efficacy in patients with relapsed SCLC [54–58]. Although in a selected patient category with PD-L1 expression, a slightly better ORR was noted.

Biomarkers in immunotherapy

PD-L1 and TMB are also emerging as biomarkers of response to immune checkpoint inhibitors in various cancer types, including SCLC [53, 59]. Most SCLC tumors seem to lack PD-L1 expression [60]. A recently conducted study speculates that only 2% of patients with SCLC exhibit amplification of the gene CD274, which encodes for PD-L1 expression, and only this small subgroup may be susceptible to ICI [61]. Currently, PD-L1 has no

clinical application in SCLC. Some evidence exists that high TMB may be associated with a response to ICI, but large phase 3 studies in patients with first-line metastasized SCLC failed to confirm this [36, 60]. The predictive role of TMB in SCLC has to be defined. The diagnosis of SCLC is made on small biopsies and the evaluation of PD-L1 in tumor tissue is challenging. Further investigation is ongoing to assess biomarkers such as TMB in tissue and in blood [62]. Liquid biopsies are less invasive for patients [63].

RECOMMENDATIONS IN CLINICAL PRACTICE

Patients with SCLC still have a poor prognosis and little progress has been made during the last few decades. Surgery for very small SCLC after adequate mediastinal staging seems feasible, but the role of adjuvant chemotherapy is still undecided. Radiotherapy is of additional value in all stages of the disease. For treatment with a curative intent, the proposed radiotherapy schedule of twice-daily irradiation, 45 Gy in 30 fractions seems feasible, and also for elderly patients. From a pragmatic perspective, once-daily radiotherapy should be considered when twice-daily radiotherapy is impractical. After completion of chemoradiotherapy, current guidelines support PCI in case of any response to chemotherapy, with most commonly a radiation dose of 25 Gy in 10 fractions of 2.5 Gy. Discussion about the benefits of PCI, both in the localized and metastasized setting is ongoing. Another option is watchful waiting with periodic brain MRI. Thoracic radiotherapy should be considered for patients with advanced disease who have any response to chemotherapy and present with symptoms such as vena cava superior syndrome, central airway compression and atelectasis of the lung.

Chemotherapy remains the mainstay of the treatment of advanced SCLC. The addition of checkpoint inhibition to the standard backbone chemotherapy has added a modest but significant improvement in outcomes, but predictive biomarkers are yet to come.

FUTURE PERSPECTIVES

Unravelling the genomics of SCLC and the subsequent discovery of biomarkers is crucial for treatment selection [64]. Whole exome sequencing may help in identifying these biomarkers and targets [65, 66]. For example, in SCLC, loss of TP53 and RB1 occurs most frequently, which results in proliferation and replication stress in SCLC and is associated with early metastasis and rapid resistance against chemotherapy [64]. Amplification of the MYC family of oncogenes occurs in 20% of SCLC and is associated with shorter survival [67]. MYC downregulation suppresses tumor growth.

The clinical relevance of biomarking SCLC lies in preferential targeting of different routes or combinations thereof, or even combining treatment modalities.

Targeting the DNA damage repair pathway

Recent preclinical studies identified predictive biomarkers of response to DNA damage repair (DDR)-targeted therapies in SCLC. Repair proteins such as poly-(ADP)-ribose polymerase (PARP), WEE1, ataxia-telangiectasia-mutated-and-Rad3-related kinase (ATR) and its major downstream effector checkpoint kinase 1 (CHK1) seems attractive to target [68]. PARP, WEE1, ATR and CHK1 prevent entry of cells with damaged or incomplete replicated DNA into mitosis and thus suppress replication stress. Inhibition of these proteins results in cell death. AZD1775 (adavosertib) is a highly selective, potent small molecule inhibitor of WEE1. This small molecule is being tested in several phase 1 and 2 studies, both alone and in combination with PARP inhibition or chemotherapy [69, 70].

Veliparib

Veliparib is a PARP-inhibitor; a small molecule that traps the DNA-repair enzyme PARP on DNA single-strand breaks and blocks its catalytic activity, thus potentially enhancing the damage to DNA caused by chemotherapy. The randomized phase 2 trial of platinum/ etoposide plus veliparib or placebo showed a median PFS of 6.1 months versus 5.5 months in favor of veliparib [71]. Recent genomic sequencing led to the identification of Schlafen 11 (SLFN11), a predictive biomarker for sensitivity to PARP inhibition in monotherapy in SCLC [72]. SLFN11 expression is high in SCLC and decreases significantly after treatment with veliparib. Prospective validation of the potential of SLFN11 as a predictive biomarker in patients treated with veliparib is warranted.

Temozolomide

Temozolomide (Temodal™) is a triazene derivative causing DNA breakage by adding an alkyl group to the guanine base of DNA. It is an oral drug, well tolerated and not very toxic, with excellent penetration into the central nervous system [73]. Beneficial effects of treatment with temozolomide in patients with SCLC were reported, especially in a subgroup associated with the presence of MGMT promoter methylation, although the difference did not meet statistical significance [74]. The RR in an unselected group was 22% in second-line, 19% in third-line, and 38% in patients with brain metastases. Temozolomide is strongly synergistic with PARP inhibition by preventing the repair of alkylated bases [75]. A phase 2 trial evaluated temozolomide with either veliparib or placebo in patients with relapsed SCLC [76]. Translational objectives included PARP-1 and SLFN11 immunohistochemical expression, MGMT promoter methylation and circulating tumor cell quantification. Four-month PFS and median OS did not differ between the two arms, but a significant improvement in ORR, which was a secondary end-point, was observed with temozolomide/ veliparib compared with temozolomide/placebo (39% versus 14%). In patients with SLFN11-positive tumors treated with temozolomide/ veliparib a significantly prolonged PFS (5.7 versus 3.6 months) and OS (12.2 versus 7.5

months) were observed, suggesting PARP-inhibitor sensitivity as a promising biomarker in SCLC. These findings were confirmed in a single-arm trial with olaparib, reporting a lesser effect in platinum-resistant disease [77].

Lurbinectedin

Lurbinectedin is a selective inhibitor of oncogenic transcription, promoting tumor cell death and normalizing the tumor microenvironment [78]. In the phase 2 basket trial, 105 patients were treated with lurbinectedin after failure of platinum-based chemotherapy [79]. Activity of lurbinectedin appeared to be greater in patients with a longer chemotherapy-free interval: ORR of 45% versus 22% in the platinum-resistant arm. Among all patients, median PFS was 3.5 months (2.6 versus 4.6 months) and the median OS was 9.3 months (5.0 versus 11.9 months). The most common toxicities were leukopenia and neutropenia. In the phase 3 ATLANTIS-trial comparing lurbinectedin/ doxorubicin with either topotecan or cyclophosphamide / doxorubicin / vincristine in second-line metastasized disease, the primary end-point OS was not met [80].

Drug resistance is often a problem of the DDR network. AXL is recognized as the key determinant in both intrinsic and acquired resistance to chemotherapeutic, immunotherapeutic and molecularly targeted agents in SCLC by epithelial-to-mesenchymal transformation (EMT) [81]. High levels of AXL and EMT predict resistance to PARP, ATR and WEE1 targeting. AXL-inhibition induces DNA damage and replication stress and promotes sensitivity to PARP and ATR inhibitors [82, 83]. In addition SLFN11 holds promise as a potential biomarker, while cells with low levels of SLFN11 were more sensitive to AXL/ATR inhibition [84].

Strategies on combinations of DDR-inhibitors or targeting multiple pathways are to be explored. Inhibitors of the DDR pathway confers a synergistic effect on immunotherapy, radiotherapy and chemotherapy; for example, temozolomide [83, 85]. PARP inhibition enhances the effect of radiotherapy in SCLC in a preclinical model [86]. A continuing challenge in SCLC is the intra-tumor heterogeneity. Blocking various routes of growth to tackle the tumor might be the answer.

Targeting the genomic and epigenomic alterations

Potential targetable genomic alterations are mutations in PTEN or RET, and amplifications of fibroblast growth factor receptor 1 (FGFR1) [87–89]. The latter are present in 6% of SCLC [89]. RET mutations are found in 1–2% of SCLC [88]. RET mutated SCLC also seems to express MYC more often. Inactivation of RB1 leads to overexpression of enhancer of zeste homolog 2 (EZH2) which promotes tumor genesis in SCLC [90]. In this process, downregulation of SLFN11 due to overexpression of EZH2 makes SCLC resistant to

chemotherapy [91]. The combination of an EZH2 inhibitor and chemotherapy, such as cisplatin or temozolomide, can circumvent resistance by preventing loss of SLFN11. Expression of EZH2 can act as biomarker and therapeutic target in SCLC.

Pazopanib

Pazopanib is a tyrosine kinase inhibitor that inhibits downstream signaling of vascular endothelial growth factor receptor (VEGFR)-1, -2 and -3. These targets are considered interesting given the importance of neoangiogenesis in SCLC and the role of VEGF overexpression in development of resistance to chemotherapy. In a multicenter, single-arm phase 2 trial, 58 patients were treated with pazopanib in second-line [92]. The platinum-refractory cohort was closed early due to futility. Median PFS and OS in cohort the platinum-sensitive cohort were 3.7 months and 8.0 months, respectively, with an ORR of 13.8%. Pazopanib is a tolerable and effective salvage treatment in patients with platinum-sensitive SCLC. However, with its modest effect, pazopanib will likely play no role except in FGFR1-amplified SCLC [93].

Alisertib

Alisertib is a selective oral Aurora kinase A (AURK) inhibitor. AURKs are mitotic regulators required for normal cell proliferation [94]. Errors in mitosis may either lead to cell death, or to aneuploidy and a mitotic dysregulator might offer a therapeutic target with relative sparing of normal cells. A phase 1–2 study in five cancer types reported a promising ORR of 21% for the SCLC arm [95]. This was further explored in a phase 2 trial randomizing between paclitaxel plus alisertib (based on preclinical evidence of synergy) or paclitaxel plus placebo, and reported a median PFS of 3.3 months for the alisertib arm, which was not significantly better than placebo (the ORR of 22% was confirmed) [96]. In a subset of patients with high MYC expression, PFS for the alisertib arm was significantly better (4.6 months versus 2.3 months), but as this analysis was not part of the protocol, this will not be further explored. In spite of this discovery of MYC as a potential biomarker in SCLC, development of alisertib was halted.

Targeting the immune system and genomic instability

Defects in the DDR pathway have been associated with enhanced responses to immune checkpoint blockade due to high TMB and genomic instability [97]. Recently, it was found that co-targeting DDR proteins such as PARP and CHK1 can increase expression of PD-L1 and antitumor immune response of anti-PD-L1 in SCLC [98, 99]. These findings suggest that DDR targeting in combination with immunotherapy could be successful.

Drug-delivery challenges

Novel drug-delivery systems such as antibody drug conjugates (ADC) bring medication in the vicinity of the tumor and should help to target tumor cells without damaging healthy cells.

Rovalpituzumab-tesirine

An initially very promising target was delta-like protein 3 (DLL3), a Notch ligand that is highly expressed in about two-thirds of SCLC [100]. Activation of Notch inhibits the growth of SCLC-cells in vitro. Rovalpituzumab-tesirine (Rova-T) is an ADC that binds to DLL3, with an ORR of 18% in the phase 1 trial, which increased to 39% in high (>50%) DLL3-expressors [101]. The subsequent phase 2 TRINITY trial selected DLL3-expressing tumors in extensively pre-treated patients (at least two lines) but found only an ORR of 12.4% and an OS of 5.7 months, with DLL3-high patients only performing slightly better than DLL3-non high patients [102]. Grade 3–5 toxicity was found in 63%, with 10% grade 5 toxicity. We may conclude that Rova-T is the first targeted agent in SCLC to target DLL3, but results are disappointing. As a result of this, the product was withdrawn and ongoing studies TAHOE (versus topotecan) and MERU (maintenance after chemotherapy) closed prematurely [103, 104].

Recently, a definition of four molecular subsets of SCLC have been proposed: acheate-scute homolog 1 (ASCL1), neurogenic differentiation factor 1 (NEUROD1), yes-associated protein 1 (YAP1) and POU domain class 2 homebox 3 (POU2F3) [105, 106]. These molecular subtypes appear to be associated with distinct expression profiles and possible therapeutic sensitivities [107]. For instance, both ASCL1-high and NEUROD1-high neuroendocrine subtypes are characterized by marked expression of insulinoma-associated protein 1 (INSM1), a marker of super-enhanced landscapes in SCLC [105]. SCLC with high NEUROD1 expression have high MYC expression [108]. High MYC expression and amplification predicts sensitivity to AURK and CHK1 inhibitors. In combination with chemotherapy, it strongly suppresses tumor progression and increases survival [67]. MYC activates Notch, so ASCL1 and NEUROD1 subtypes could benefit from DLL3 inhibitors such as Rova-T. The combination of Rova-T and immunotherapy, however, was not well tolerated despite antitumor activity in third-line and beyond [109]. Subtypes with low ASCL1, NEUROD1 and POU2F3 expression act as inflamed SCLC and can benefit from the addition of immunotherapy to chemotherapy [110].

CONCLUDING REMARKS

The histopathology and tumor biology of SCLC is complex. Simple strategies to target the tumor are not successful in achieving a long survival advantage. Several approaches have the potential to overcome the well-known treatment failures of the last decades.

Firstly, recent studies have shown progress in finding biomarkers to serve as targets for treatment. Extensive exome sequencing in patients with SCLC is the future to create a landscape of predictive biomarkers in SCLC. Epigenetic alterations, gene amplifications and mutations can act as biomarkers in this context. Consequently, biomarker-driven patient selection is needed to stratify patients for treatments. Distinct molecular subtypes appear to be associated with therapeutic sensitivities. The key to success lies in the treatment combination or targeting dual pathways that have additional or synergistic effects. Overcoming intra-tumor heterogeneity is an extra hurdle where combination therapy, concomitantly or sequentially, is probably a “conditio sine qua non”. Secondly, it is expected that adding other treatment modalities, such as radiotherapy or immunotherapy, to biomarker-driven drug combinations will have synergistic effects to overcome resistance mechanisms. Lastly, novel drug-delivery systems should help to target tumor cells while preventing deleterious effects due to interaction with healthy cells.

With possible biomarkers having been discovered, the design of future trials should allow the study of a targeted treatment in a biomarker enriched population. Importantly, referrals of patients for clinical trials with biomarker-selected targeted treatments is warranted to improve the prognosis for patients with SCLC.

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Chapter 3

Small-Cell Lung Cancer: Is the Black Box Finally Opening Up?

Editorial

Birgitta I. Hiddinga
Klaas Kok

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Small-cell lung cancer (SCLC) is an aggressive cancer that originates from the neuroendocrine crest. It comprises about 15% of lung cancers and has a dismal prognosis [1]. The 5-year survival has hardly increased in recent decades and is now 6% [1]. In the last decade, few new treatment modalities were implemented, and treatment with chemotherapy and radiotherapy have remained the mainstay of therapy [2]. Thus, in clinical practice we are still in need of targetable biomarkers to treat patients with SCLC in order to substantially improve prognosis in this patient category. In this Special Issue on "Targeted therapy for Small Cell Lung Cancer" we aim to open up the black box that SCLC used to be, trying to reveal targetable biomarkers, as well as biomarkers that can stratify patient groups for more effective treatments, in order to improve the prognosis for this devastating disease. At the genomic level, SCLC is characterized by a high mutational burden with close to, or even more than, ten somatic mutations per megabase and high chromosomal instability [3,4]. TP53 and RB1 are the most frequently mutated genes in SCLC, resulting in loss of function for these genes [5]. Despite this high mutational burden, very few targetable driver mutations are observed. Mutations in activated tyrosine kinases that can subsequently be targeted by specific inhibitors (TKIs) are a well-known phenomenon for adenocarcinoma of the lung, but are rare in SCLC [6,7]. In the treatment of SCLC, these TKIs have failed. The addition of immunotherapy to chemotherapy in the first line treatment of advanced stage SCLC, instigated by the high mutational burden, improved progression free survival by only two months [8]. Of special interest when treating with immunotherapy, biomarkers such as PDL1 and TMB did not prove as reliable biomarkers to predict the patient population that would benefit from treatment with immunotherapy [9]. Recently, four subsets of SCLC have been described: achaete-scute homolog 1 (ASCL1), neurogenic differentiation factor 1 (NeuroD1), yes-associated protein 1 (YAP1) and POU domain class 2 homebox 3 (POU2F3). These molecular subtypes appear to be associated with distinct expression profiles and have different therapeutical sensitivities [10]. Most cases of SCLC express INSM1, a marker of both ASCL1-high and NEUROD1-high neuroendocrine subtypes and super-enhanced landscapes in SCLC [11]. A small fraction of SCLC tumors are INSM1-low, ASCL1-low and NeuroD1-low. These tumors lack neuroendocrine markers and appear to fall into discrete YAP1-high and POU2F3-high subtypes [12].

THE SEARCH FOR NOVEL TARGETED TREATMENTS

Both Schultze et al. [13] and Lum et al. [14] give a general overview of the attempts that have been pursued in the past years to develop new targeted therapies for SCLC, although with slightly different angles of approach. After introducing the mutational profile that characterizes SCLC, Schulze et al. [13] describe why current diagnostic tools have not been successful. Subsequently they present a broad perspective on possible future

options for molecular-targeted therapy, especially focusing on potential biomarkers for treatment. The authors highlight several surface markers, apoptotic markers, genetic alterations and vascular targets that may act as promising targets and review the status of a number of clinical and preclinical studies. Some of the targeted treatments did not improve the outcome and have already been withdrawn. Several additional novel targets need further evaluation and prospective validation before entering the clinic. In their opinion, the data on aurora kinase inhibitors and PARP inhibitors present the most promising developments. Aurora kinases phosphorylate key components of the cell cycle, whereas PARP1 is an important enzyme in DNA damage repair pathways.

Lum et al. [14] review the genomic structure of SCLC and describe the further subclassification of SCLC into four distinct molecular subtypes: ASCL1, NeuroD1, YAP1 and POU2F3, for which there may be distinct therapeutic targets. They also address an additional complicating factor in the treatment of SCLC, e.g., inter- and intratumor heterogeneity. Intertumor heterogeneity, also a reflection of the above-mentioned subtyping, demands for the efficient stratification of patients with respect to the available treatment strategies. Intratumor heterogeneity may result in not all tumor cells of a patient responding equally to the same treatment. In the absence of oncogenic driver gene mutations that can be targeted, many strategies focus on interfering with crucial pathways and cellular processes. In their extensive overview, Lum et al. group the current therapeutic strategies into five major target categories: development and regulatory pathways, DNA damage and repair (DDR) pathways, epigenetic processes, cell cycle, and the immunotherapy field. Evolving classification of SCLC molecular subtypes is being anticipated as a major development and may help to further stratify patients. In the future, efficient treatment of patients will also depend on the availability of biomarkers that efficiently select patients that respond to a specific treatment. In this respect, real future-changing perspectives will be expected in the field of liquid biopsies. Liquid biopsy analyses could potentially help to stratify patients, for longitudinal monitoring of disease, and for early detection of progression. As reviewed by Lum et al., for SCLC, the focus has been on circulating tumor cell counts and the analysis of circulating cell free DNA. The authors conclude that improved understanding of this devastating disease is underway to underpin the genomics, molecular profiling, resistance mechanisms, and novel therapies in SCLC. They see the further subclassification of SCLC as a major and important step in the development of personalized targeted therapies, but at the same time recognize the high mutation rate and the intratumor heterogeneity as threat that needs to be dealt with.

TARGETING REPLICATION STRESS

A characteristic feature of SCLC is the high level of replication stress (RS), most likely correlated with the high mutation rate. A high level of RS can cause mitotic catastrophe and ultimately cell death [15]. To counteract this, the tumor cells need a very robust DNA damage response (DDR) system or a very active replication stress response (RSR) pathway. These might be considered as SCLC's weak spots. After providing a short review on the principles of RS, Bian et al. [16] summarize the source of RS in SCLC and review the strategies to take advantage of this by either increasing the RS or blocking the DDR system in the tumor cells. Of several inhibitors that target the replication stress pathway, PARP1 and WEE1 appear to be the most promising, based both on preclinical and clinical studies. DNA repair targets encompass ATM, RAD51, but also several genes involved in cell cycle control such as PLK1 and aurora kinases. Although several of the inhibitors developed against these genes have shown some effect as monotherapies, they may be even more effective in combination with a second therapy. Drug-resistance is often due to the complexity of the DNA damage response network, so combinations of replication stress inducers with other therapeutics are explored. As an example, Bian et al. review how targeting PARP appears to increase the sensitivity towards immunotherapy by increasing PD-L1 expression.

TARGETING THE MESENCHYMAL-EPITHELIAL TRANSITION (MET) PATHWAY

Yet another angle to develop new therapies is discussed by Harby-Werbin et al. [17]. They review the role of the hepatocyte growth factor (HGF)/mesenchymal–epithelial transition (MET) pathway, the activation of which is, amongst others, associated with chemoresistance in SCLC. In metastasized SCLC, overexpression of nuclear topoisomerase1 correlated with an increased overexpression of MET. As a proof-of-concept, patients with SCLC were treated with MET inhibitors. Although the outcome of these studies suggested a role for MET inhibition in SCLC, in clinical trials the successes have been disappointing. As MET is acting as an epitope, it could be recognized by cytotoxic CD8+ T cells, eliciting an activation toward tumor cells expressing MET. This is the rationale for combination therapy of a MET-inhibitor with immunotherapy. The authors indicate the importance of assessing the tumor tissue for at least MET-expression and/or MET-mutations, HGF expression and immune infiltrate evaluation as potential biomarkers to select patients that will profit from a combined treatment of MET inhibitors and immunotherapy.

FOCAL ADHESION KINASE INHIBITION

In an original article on the expression and activation of focal adhesion kinase (FAK) Aboubakar Nana et al. [18] evaluated the potential role of FAK as a prognostic marker. FAK is a tyrosine kinase found in focal adhesions, intracellular complexes that are formed following engagement of the extracellular matrix by integrins. FAK-dependent activation of several downstream pathways has been implicated in multiple cellular processes, including cell migration, growth factor signaling, cell cycle progression and cell survival. Consistent with its role in cell migration and angiogenesis, increased abundance or activation of FAK is found in a variety of cancers. As the prevalence of FAK expression in lung cancers was unknown, the authors analyzed 95 non-small cell lung carcinoma (NSCLC), 105 SCLC and 37 normal lung tissue specimens for FAK and phospho-FAK protein levels. FAK abundance was significantly higher in the more malignant SCLC than in NSCLC, which in turn had significantly higher expression levels than normal lung tissue. The phosphor-FAK fraction appeared to be significantly higher in SCLC as compared to NSCLC as well, both in the cytoplasmic and the nuclear compartment. However, neither the expression level of FAK nor that of phospho-FAK correlated with recurrence-free and overall survival in NSCLC and SCLC patients. The authors conclude that FAK-expression cannot act as a prognostic biomarker in SCLC but may be interesting in terms of targeted therapy. However, FAK could still be a biomarker to select patients that may respond to FAK inhibitors. This latter option is further explored in a review by Aboubakar Nana et al. [19], that starts out with an in-depth description of the role of FAK in multiple cellular processes and how its overexpression and activation is a recurrent factor in solid tumors. As silencing of FAK augments apoptosis in cancer cells, synergistic effects of combinations with other therapies like chemotherapy, immunotherapy or radiotherapy may improve patient outcomes. Of special interest, is the role of FAK in immunotherapy as FAK activity was elevated and correlated with high levels of fibrosis and poor CD8+ cytotoxic T cell infiltration. FAK inhibition substantially limited tumor progression, resulting in a delay in tumor progression, associated with markedly reduced tumor fibrosis and a decreased number of tumor-infiltrating immunosuppressive cells. This raises the question whether FAK inhibition added to immunotherapy is able to result in a better prognosis in SCLC. The reviews discussed above have all shown some promising preclinical results with respect to targeted therapy. However, in subsequent clinical studies these therapies often show disappointing results.

DRUG DELIVERY SYSTEMS

A major hurdle is delivery of the drug to the tumor cells without causing detrimental damage to healthy tissues. Tumor-targeting drug conjugates are an emerging novel therapeutic approach in SCLC, and in their review Deneka et al. discuss the possibilities and ongoing studies regarding delivery systems to bring therapy into the vicinity of the tumor [20]. Antibody–drug conjugates (ADC), radioimmunoconjugates (RICs), small molecule–drug conjugates (SMDCs) and polymer–drug conjugates (PDCs) are the major delivery systems. Several benefits are clear: better prevention of off-target toxicity and trying to allow an effective dosage of the drug to the tumor with a tolerable toxicity. A challenge is to elude systemic toxicity to optimize the response on the tumor. All options need biomarkers to target for effective use of drug conjugates. Several questions are of importance to the clinician. Can we deliver these targeted treatments together with radiotherapy, or in combination with other drugs, for example immunotherapy? In the near future, radionuclides linked to tumor-targeting antibodies may play a prominent role in tumor imaging and optimizing the diagnostic path that patient will follow. Limitations are in the nature of their radioactive payloads.

CONCLUDING REMARKS

In reading the contributions to this special issue, one might conclude the glass is half empty, and that the main conclusion is that SCLC is a resilient tumor for which treatment options are still distant. Although a number of preclinical trials have shown exiting results, subsequent clinical trials have often yielded disappointing outcomes. On the other hand, one could also conclude that the glass is half full, as an enormous amount of effort has been dedicated to these studies, and that recent studies do show progress in finding better treatments for this disease. Some new targets have been identified, with the aurora kinases as good example, that could potentially lead to better treatments. It could well be that it is not a shortage of therapeutic targets that is the main problem, but instead the knowledge on how and when to use them. Three main directions that emerge from the contributions could potentially improve outcomes in patients with SCLC. Firstly, we need more tools to accurately stratify patients for specific treatments. The intratumor heterogeneity may be the main problem in the treatment of SCLC, as it results in no single treatment being effective for the majority of patients. The outcome of clinical studies may improve considerably if tools were available to carefully preselect patients that could potentially benefit from a specific treatment. The further classification of SCLC into four subgroups is a first step in this process [12]. MYC amplification has already been suggested to predict sensitivity towards aurora kinase inhibitors [21]. Secondly, the solution may be lying in combination therapy rather than

monotherapy. Targeting dual pathways can be more efficacious to stop proliferation of tumor cells. The included reviews give ample suggestions where a novel drug may actually have a synergistic effect on the response to one of the classic treatments. Thirdly, novel drug delivery systems should help to specifically target the tumor cells while preventing deleterious effects due to the interaction with healthy cells. Taken together in this Special Issue, several routes have been discussed that may lead to new promising treatments for SCLC.

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Addendum

Current developments in the treatment of SCLC

Birgitta Hiddinga

INTRODUCTION

Since the publication of Chapter 2 in 2021, additional studies on chemoimmunotherapy in first-line metastasized SCLC reported outcomes and new studies have been initiated [1 – 8] (Table 1). Some prospective single arm studies with platinum/etoposide and anti-PD-(L)1 in real-world settings reported comparable outcomes on progression free survival (PFS) and overall survival (OS) [6 – 8]. A study conducted in Spain included patients with performance status > 2 and patients with brain metastases [6]. All studies showed the benefit of immunotherapy, although in fragile patient groups with performance status > 2 and/or with brain metastases adverse events were more obvious, impacting survival. Despite a benefit of 1.8 months, the Netherlands did not consider immunotherapy for advanced SCLC to be eligible for insurance reimbursement, although survival rates after one and three years showed better results for the combination with chemoimmunotherapy than chemotherapy alone.

Table 1. First-line chemoimmunotherapy for SCLC

Study [ref]	Checkpoint inhibitor	Patients	mPFS (mo) HR	mOS (mo) HR	1y OS rate	3y OS rate
Impower133 [1]	Atezolizumab	403	5.2 HR 0.77	12.3 HR 0.76	52% vs 38.2%	
CASPIAN [2]	Durvalumab	805	5.1 HR 0.80	12.9 HR 0.71	54% vs 40%	17.6% vs 5.8%
KEYNOTE 604 [3]	Pembrolizumab	453	4.8 HR 0.70	10.8 HR 0.76	45% vs 40%	15.5% vs 5.9%
ASTRUM-005 [4]	Serplulimab	585	5.7 HR 0.48	15.4 HR 0.63	61%	
CAPSTONE-1 [5]	Adebrelimab	462	5.8 HR 0.67	15.3 HR 0.72	63% vs 52%	
Single arm prospective chemotherapy plus immunotherapy in metastasized SCLC						
Imfirst [6]	Atezolizumab	155	6.2	10.0		
Tamiya [7]	Atezolizumab	207	5.1	15.8		
MAURIS [8]	Atezolizumab	154	5.5	10.7	41.9%	

SCLC: small cell lung carcinoma; mPFS: median progression free survival; HR: hazard ratio; mOS: median overall survival; 1y OS rate: 1 year overall survival rate; 3y OS rate: 3 year overall survival rate; mo: months.

COMBINATION OF TREATMENT MODALITIES

Now that immunotherapy is applied in the lower stages of the disease, it will be of vital importance to know if the addition of immunotherapy to chemoradiotherapy will lead

to better results in limited stage SCLC [9 – 14] (Table 2). In anticipation of the results of ADRIATIC, namely treatment with adjuvant durvalumab after chemoradiotherapy [9], other studies are still ongoing. The combination of chemoimmunotherapy with thoracic radiotherapy seems feasible based on some retrospective series and is now prospectively being explored in limited stage SCLC (Table 2). In practice, the expected toxicity radiation pneumonitis turns out to be manageable [15].

Table 2. Trials in limited stage SCLC with chemoradiotherapy and immunotherapy

Study [ref]	Trial design	Treatment	Patients	Endpoints
ADRIATIC [9]	Phase 3, RCT, double-blind Adjuvant ICI	1) Durvalumab + placebo 2) Durvalumab + tremelimumab 3) Placebo + placebo	724	PFS, OS in endpoints Adriatic
SURPASS [10]	Phase 2, 3 RCT, double-blind Adjuvant ICI	1) Sugemalimab 2) Placebo	346	PFS
ACHILES [11]	Phase 2 Adjuvant ICI	1) Atezolizumab 2) Observation	212	2-y OS rate
NCT04189094 [12]	Phase 2, open label Concomitant ICI	1) Platinum / etoposide + sintilimab + TRT 2) Platinum / etoposide + TRT	140	PFS
NRG-LU005 [13]	Phase 3, open label Concomitant ICI	1) Platinum / etoposide + thoracic radiotherapy 2) Platinum / etoposide + thoracic radiotherapy + atezolizumab + maintenance	545	OS
Park [14] NCT03585998	Phase 2, single arm Concomitant ICI	Platinum / etoposide + durvalumab + TRT	51	mPFS 14.4 mo mOS NR 2-y PFS rate: 42% 2-y OS rate: 67.8%

SCLC: small cell lung cancer; RCT: randomized controlled trial; ICI: immune checkpoint inhibitor; TRT: thoracic radiotherapy; mPFS: median progression free survival; mOS: median overall survival; mo: months; 2-y PFS rate: 2 year progression free survival rate; 2-y OS rate: 2 year overall survival rate; NR: not rated.

As multimodality therapy has proved to lead to better results than applying monotherapy, the new strategy of adding radiotherapy to chemotherapy or chemoimmunotherapy in metastasized setting SCLC were explored [16 – 30] (Table 3). So far, only two studies mention the assessment of biomarkers [18, 26].

Table 3. First-line metastasized SCLC adding thoracic radiotherapy to chemoimmunotherapy

Study [ref]	Trial design	Treatment	Patients	Endpoints
RAPTOR [16] NCT04402788	Phase 2 – 3, RCT	1) Atezolizumab after response on chemotherapy and atezolizumab 2) Atezolizumab + (extra) thoracic radiotherapy after response on chemotherapy and atezolizumab	138 phase 2 186 phase 3	Phase 2: PFS Phase 3: OS
TRIPLEX [17] NCT05223647	Phase 3, RCT	1) Platinum / etoposide + durvalumab + TRT 2) Platinum / etoposide + durvalumab	302	Change in 1-year OS
TREASURE [18] NCT04462276	Phase 2 Maintenance after atezolizumab + platinum + etoposide	1) Atezolizumab 2) Atezolizumab + radiotherapy	104	OS
SAKK15/19 [19] NCT04472949	Phase 2	Induction phase: durvalumab + platinum / etoposide. Arm 1. CR, PR or SD: maintenance phase: durvalumab + TRT Arm 2. PD: follow up	46	PFS
Chen [20]	Phase 2, single arm	After platinum / etoposide sequential TRT + adebrelimab	31	Median PFS 7.5 mo ORR 50% DCR 87.5%
NCT04562337 [21]	Phase 2	Platinum / etoposide + SHR1316 (adebrelimab) + TRT	67	OS
CASPIAN-RT [22] NCT05161533	Phase 2 Maintenance after platinum / etoposide + durvalumab	TRT (hypofractionated) + durvalumab	50	PFS
NCT05092412 [23]	Phase 2	Platinum / etoposide + durvalumab + LDRT 5x3Gy	30	PFS
MATCH [24] NCT04622228	Phase 2	Platinum / etoposide + atezolizumab + LDRT 5x3Gy	56	ORR
NCT04951115 [25]	Phase 2	Platinum / etoposide + durvalumab + SBRT (6Gy on multiple intrathoracic sites)	42	Adverse events ≥ grade 3 Efficacy of radiation measured by change in disease response

Study [ref]	Trial design	Treatment	Patients	Endpoints
Perez [26]	Phase 1 and 2	Nivolumab + ipilimumab + TRT after chemotherapy	21	Median PFS 4.5 mo Median OS 11.7 mo
NCT05403723 [27]	Phase 1B	Induction phase: Platinum / etoposide + durvalumab Arm 1. CR, PR: maintenance. Arm 2. SD, PD: SBRT 6x5Gy + maintenance	50	Adverse events
Peng [28]		Chemotherapy + durvalumab or atezolizumab Arm 1. TRT Arm 2. No TRT	114	Arm 1, median PFS 9.5 mo Arm 2, median PFS 7.2 mo HR 0.59
Welsh [29]	Phase 1 After platinum / etoposide	Concomitant pembrolizumab + TRT	38	mPFS 6.1 mo mOS 8.4 mo
NCT03923270 [30]	Phase 1 After platinum / etoposide	1) Durvalumab + tremelimumab 75 mg + TRT 2) Durvalumab + Olaparib + TRT 3) Durvalumab + tremelimumab 300 mg + TRT 4) Durvalumab + TRT	25	Adverse events Phase 1B: PFS

SCLC: small cell lung cancer; RCT: randomized controlled trial; TRT: thoracic radiotherapy; PFS: progression free survival; mPFS: median progression free survival; OS: overall survival; mOS: median overall survival; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; mo: months; DCR: disease control rate; LDRT: low-dose radiotherapy; SBRT: stereotactic body radiotherapy.

BIOMARKERS

In search of biomarkers in SCLC predicting response to immunotherapy, in the Impower133 RNA-sequencing was used to analyze gene expression in long-term survivors treated with first-line carboplatin and etoposide with or without atezolizumab [31]. Long-term survivors were defined as surviving 18 months or more after randomization. Long-term survivors were more often treated with atezolizumab plus chemotherapy (33.5%) than placebo plus chemotherapy (20.4 %). PD-L1 expression was most frequently observed on tumor-infiltrating immune cells, with limited expression on tumor cells. The overlap of patients with high PD-L1 and a high tumor mutational burden (TMB) was limited. On the contrary, in a patient group treated with nivolumab or nivolumab plus ipilimumab, a high TMB was associated with a complete or partial response rather than stable disease or progressive disease, which is suggestive of TMB being a predictive

factor [32]. These findings suggest that TMB and PD-L1 status should not be used for patient treatment decisions, as neither biomarker is predictive of outcome. The current lack of identification of a biomarker for checkpoint inhibitors in SCLC, warrants additional studies to further evaluate potential biomarkers and associations with outcomes.

Biomarkers in blood samples are gaining more and more attention [33, 34]. Recently published papers on cell-free nucleosome profiling in plasma can improve our knowledge of the biology of tumors, but have also detected certain responses to therapy [35, 36]. In ADRIATIC, ACHILES and NRG-LU005, biosamples are collected to validate circulating tumor DNA (ctDNA) as an early endpoint of clinical outcome [9, 11, 13].

SCLC CLASSIFICATION INTO FOUR MOLECULAR SUBTYPES AND ITS THERAPEUTIC VULNERABILITY

The proposed classification of SCLC into four molecular subtypes: acheate-scute homolog 1 (ASCL1), neurogenic differentiation factor 1 (NEUROD1), yes-associated protein 1 (YAP1) and POUdomain class 2 homebox 3 (POU2F3) – SCLC-A, -N, -P, and -Y, respectively – makes sense – and is helpful - as each has its own therapeutic sensitivity and is therefore susceptible to certain medication [37](Table 4). The prognostic relevance of these subtype-specific proteins is an area of research in small biopsies and in surgical specimens [38, 39].

SCLC-A and SCLC-N tumors have been reported to be associated with elevated expression of neuroendocrine markers, such as chromogranin A and synaptophysin [40, 41], whereas SCLC-P and SCLC-Y tumors with a higher RE1 silencing transcription factor (REST) – a repressor of neuroendocrine genes – result in a less neuroendocrine phenotype [42, 43]. Synaptophysin was often expressed in the ASCL1 and NEUROD1 subtypes and was associated with less tumor-associated CD8+ T-cells and a “desert” immunophenotype. In one study, immunohistochemical staining of defined ASCL1, NEUROD1, POU2F3, and YAP1 dominant molecular subtypes were found in 78.2%, 5.6%, 7%, and 2.8% of the tumors, respectively; 6.3% of the tumors were negative for all four subtype markers [38]. The low expression of ASCL1, NEUROD1 and POU2F3 resulted in the renaming of SCLC-Y to SCLC-I, the “inflamed” immunophenotype which had more CD8+ T-cells, and this subtype may well benefit from adding immunotherapy to chemotherapy [43 – 46].

Each subtype has targets that are most abundant and they can serve as biomarkers to enable targeted treatment (Table 4). Both SCLC-A and SCLC-N are characterized by marked expression of insulinoma-associated protein 1 (INSM1), Schlafen 11 (SLFN11) and MYC [41]. High MYC expression and amplification in SCLC-N predict a sensitivity to

aurora kinase (AURK) inhibitors and checkpoint kinase 1 (CHK1) inhibitors [47, 48]. In combination with chemotherapy, tumor progression is strongly suppressed and survival increases [49]. As SCLC is characterized by a high TMB and targeting the DNA damage response pathway genes is considered an effective method for treatment. In the phase 2 single arm in relapsed SCLC, the ATM/ATR inhibitor berzosertib plus topotecan reached an ORR of 36% [50]. Randomization in DDRiver SCLC 250, berzosertib plus topotecan versus topotecan alone will reveal whether this leads to a better treatment option in second line SCLC [51]. Furthermore, in cell lines of SCLC-N, we see a high expression of somatostatin receptor 2 (SSTR2), acting as a target for somatostatin analogues [44]. Phase 1 studies are initiated to test SSTR in combination with first line treatment in ES-SCLC [2].

Table 4. Therapeutic vulnerability of four SCLC subtypes

SCLC-A	SCLC-N	SCLC-P	SCLC-Y
DLL3 BCL2 EZH2 INSM1 LSD1 L-MYC NFIB RET SOX2 Synaptophysin Chromogranin	AURK MYC CHK1 INSM1 LSH1 NFIB TrkB	AURK MYC IGF-1R SLFN11 ATM EMT	mTOR RB1 SOX9 PLK LAG3
DLL3 CREBBP BCL2 HDAC inhibitor LSD1 inhibitor	AURKA inhibitor cMYC inhibitor CHK1 inhibitor Arginine deprivation	Arginine deprivation PARP inhibitor CHK1 IGF-1R inhibitor Nucleoside analogues	ICI mTOR inhibitor PLK inhibitors CDK4/6 inhibitor

Delta-like protein 3 (DLL3) is an inhibitory Notch receptor implicated in tumorigenesis. It is a cell surface marker driven by ASCL1, and is thus more highly expressed in SCLC-A tumors, and lowest in SCLC-P and SCLC-I [44]. Rovalpituzumab tesirine (Rova-T) is a DLL3-specific antibody-drug conjugate (ADC) [53]. As MYC activates Notch, both ASCL1 and NEUROD1 subtypes could benefit from DLL3 inhibitors such as Rova-T, but, unfortunately, Rova-T failed in all settings treating SCLC: maintenance after first line chemotherapy, in second line versus topotecan and in third line and later, even in selected DLL3-high patient groups [54 – 56]. Furthermore, the combination of Rova-T and immunotherapy was not well tolerated despite antitumor activity in third-line and beyond [57].

The subtype SCLC-P seems more vulnerable to poly-(ADP)-ribose polymerase (PARP) inhibitors, although the expression of SLFN11 was highest in SCLC-A and only modest

in SCLC-P [58, 59, 44]. SLFN11 expression in ctDNA can be a prospective biomarker for treatment with PARP inhibitors (PARPi), but this needs to be further investigated [60]. In second line, temozolomide plus a variety of PARPi is explored. Only a few studies aim at selecting biomarkers, for example, SLFN11 positive patients are eligible for atezolizumab plus PARPi talazoparib in a maintenance setting [61]. The phase 2 study in relapsed SCLC SUKSES-B2 involves recruiting 28 patients with ATM deficiency, SLFN11 positive, or POU2F3 positive, for treatment with olaparib and bevacizumab [62]. In addition to PARPi, SLFN11 predicts sensitivity to chemotherapy and lurbinectedin. Lurbinectedin is still being investigated to verify whether earlier use would offer greater benefit [63]. The IMforte study will reveal if a potential synergy with immunotherapy exists and whether lurbinectedin is the appropriate maintenance partner with a checkpoint inhibitor [64]. Other combinations are explored, for example lurbinectedin in combination with berzosertib, which is also available in LCNEC [65].

Since SCLC-I acts as the inflamed subtype, it would be important to know whether this subtype performed better in studies with first line chemoimmunotherapy compared to chemotherapy alone. Retrospective staining of some of the largest sets of SCLC until now is currently available [66, 45, 67, 44] (Table 5).

Table 5. Retrospective staining of immunotherapy studies in SCLC

Study [ref]	Number	SCLC – A	SCLC – N	SCLC – P	SCLC – Y
George [66]	110	36%	31%	16%	17%
Checkmate032 [45]	156 nivolumab	32% (49)	30% (47)	14% (21)	25% (39)
	130 nivolumab + ipilimumab	31% (40)	36% (47)	11% (14)	22% (29)
CASPIAN [67]	104	37%	44%	12%	11%
Impower133 [44]	276	51%	23%	7%	18%

In Checkmate032, comprehensive bulk RNA sequencing was performed on 286 pretreatment biopsy samples, including 156 samples after treatment with single-arm nivolumab and 130 treated with nivolumab plus ipilimumab [45]. Other biomarkers were explored resulting in increased survival when high CD8+ T cell infiltration (HR 0.51 for nivolumab and HR 0.70 for nivolumab plus ipilimumab) and high MHC-I expression were found (HR 0.59 for nivolumab and HR 0.87 for nivolumab plus ipilimumab). In the CASPIAN study, among the four subtypes, the median OS in the chemotherapy plus durvalumab group was highest in the SCLC-Y or SCLC-I group with 17.6 months, suggesting a subgroup predicting better response to immunotherapy, although the sample size is small [67]. The distribution of subtypes in Impower133 varies somewhat [44, 68]. The benefit of atezolizumab, however, was observed in all subtypes.

REFINING DELIVERY STRATEGIES?

ADCs, radioimmunoconjugates (RICs), small molecule–drug conjugates (SMDCs) and polymer–drug conjugates (PDCs) are the major delivery systems. Several benefits are clear: better prevention of off-target toxicity and the option to administer an effective dosage of the drug to the tumor with a tolerable toxicity. The challenge is to avoid systemic toxicity to optimize the response on the tumor. All the various options would benefit from biomarkers to aim for the more effective use of drug conjugates.

As we have seen in the development of Rova-T, next generation ADCs have to be designed to overcome certain limitations: enhanced antibody formats with new linkage technologies, improved stability profiles, and an optimized drug-antibody ratio aim to improve pharmacokinetics and expand the therapeutic window [69]. Moreover, ADC payloads can prime dendritic cells directly, suggesting that ADC and immunotherapy can enhance their efficacy [70]. Even immune-PET imaging can be of assistance to provide information relating to protein expression, for example DLL3 expression, in order to better select patients for DLL3-targeted therapies [71]. This study is open to all high grade neuroendocrine tumors.

Seizure-related homolog 6 (SEZ6) is a downstream target of ASCL1 and provides a clinical target for novel ADCs [72]. SEZ6 is predicted to be involved in neuronal maturity and plasticity and is mostly expressed in SCLC-P [73]. ADCs for SEZ6 are being developed, namely ABBV-706 and ABBV-011, or combined with PD-1 inhibitor budigalimab [74, 75]. Both compounds will be available for other neuroendocrine tumors, and hopefully enlarge therapeutic arsenal in all NETs.

Other ADCs are the pegylated conjugate of SN38 (conjugate of irinotecan) combined with PARPi rucaparib [76], the ATR inhibitor elimusertib (BAY-1895344) plus standard chemotherapy in second line [77] and two ADCs against trophoblast cell surface antigen 2 (Trop2) Sacituzumab govitecan [78] and SKB264 -ADC's [79]. The latter is developed for NSCLC, but will be available for NECs in the near future. Sacituzumab govitecan reached an ORR of 14%, mPFS 3.7 months and mOS 7.5 months in SCLC.

EP0057 is a nanoparticle drug conjugate (NDC) camptothecin, a potent topoisomerase I inhibitor, which will be evaluated in combination with olaparib in phase 2 [81].

HOW DO WE RAISE THE TAIL OF THE SURVIVAL CURVE?

Although the combination of PD-(L)1 inhibition with CTLA4 inhibition or anti-TIGIT (Tiragolumab) failed in SCLC [2, 82], alternatives are explored, including different types of immunotherapy [57, 82 – 100] (Table 6).

Table 6. New agents in immunotherapy in SCLC

Study [ref]	Trial design	Treatment	Patients	Endpoints
Studies with new immunotherapies in limited disease SCLC				
NCT04308785 [83] Anti-TIGIT	Phase 2, RCT, double-blind Adjuvant ICI + anti-TIGIT	1) Atezolizumab + tiragolumab 2) Atezolizumab + placebo	242	PFS in ITT population
AdvanTIG-204 NCT04952597 [84] Anti-TIGIT	Phase 2, RCT Concomitant chemoradiotherapy + anti-PD-1 + anti-TIGIT	1) Platinum / etoposide + ociperlimab + tislelizumab + TRT 2) Platinum / etoposide + tislelzumab + TRT 3) Platinum / etoposide + TRT	126	PFS
Studies with new immunotherapies in metastasized SCLC				
SKYSCRAPER-02 [82] NCT04256421	Phase 3, RCT, double-blind, anti-PD-L1 + anti-TIGIT	1) Platinum / etoposide + tiragolumab + atezolizumab 2) Platinum / etoposide + placebo + atezolizumab	490	OS 13.6 mo vs OS 13.6 mo
Malhotra [57]	Phase 1, 2 RCT, open label Heavily pretreated SCLC	1) Nivolumab + Rova-T 2) Nivolumab + Rova-T + ipilimumab	42	ORR 30% Not well tolerated
DeLLphi-300 NCT03319940 [85]	Phase 1, 2 open label in relapsed or refractory SCLC BiTe targeting DLL3	AMG757 Tarlatamab	107	mPFS 3.7 mo mOS 13.2 mo ORR 23.4% mDOR 12.3 mo DCR 51.4%
DeLLphi-301 NCT05060016 [86]	Phase 2 RCT open label in relapsed or refractory SCLC (3rd line)	AMG757 Tarlatamab 1) low dose 2) high dose 3) dose expansion	222	ORR Adverse events Serum concentrations

Study [ref]	Trial design	Treatment	Patients	Endpoints
DeLLphi-303 [87] NCT05361395	Phase 1B, single arm in first line	AMG757 (tarlatamab in different doses) + carboplatin/ etoposide + atezolizumab	340	Dose finding
DeLLphi-304 [88] NCT05740566	Phase 3, RCT open label in relapsed or refractory SCLC (2nd line)	1) tarlatamab 2) investigator choice: lurbinectedin or topotecan or amrubicin	700	OS
NCT04885998 [89]	Phase 1 in relapsed or refractory SCLC	Tarlatamab + AMG404 (Zeluvalimab = anti-PD-1)	23	Dose finding AMG404
NCT04750239 [90] Halted because of high grade cytokine release syndrome	Phase 1 BiTe with tetravalent structure against GD2 and CD3	Nivatrotamab		
NCT04429087 [91]	Phase 1 BiTe targeting DLL3	BI-764532	193	
NCT05652686 [92]	Phase 1 BiTe targeting DLL3 and CD47	PT217	58	
NCT05461287 [93]	Phase 1 BiTe targeting DLL3 and CD3	QLS31904	290	
NCT05619744 [94]	Phase 1 multispecific antibody against DLL3 and CD3/ CD137	RO7616789	168	
NCT04471727 [95]	Phase 1, 2 TriTAC	Phase 1: HPN328 Phase 2: HPN328 + atezolizumab	162	
NCT05507593 [96]	Phase 1 in relapsed or refractory SCLC	DLL3-CAR-NK cells	18	Dose finding, adverse events
NCT03392064 [97] Suspended	Phase 1 single arm in relapsed or refractory SCLC. CAR-T targeting DLL3	AMG119	6	Safety, tolerability, efficacy
NCT05680922 [98]	Phase 1 CAR-T targeting DLL3	LB2102	41	Safety, dose finding
NCT05620342 [99]	Phase 1 CAR-T targeting GD2 antigen	iC9.GD2.CAR. IL-15 T (IL-15, and iCaspase9 Safety Switch)	24	

Table 6. Continued

Study [ref]	Trial design	Treatment	Patients	Endpoints
NCT05026593 [100]	Phase 2, RCT, open label Anti-LAG-3	1) Platinum / etoposide + sintilimab + IBI110 2) Platinum / etoposide + sintilimab	60	PFS Adverse events

SCLC: small cell lung cancer; TIGIT: T-cell immunoglobulin and ITIM domain; RCT: randomized controlled trial; PFS: progression free survival; ITT: intention-to-treat; TRT: thoracic radiotherapy; mo: months; BiTE: Bispecific T-cell engager; DLL3: delta-like protein 3; mPFS: median progression free survival; mOS: median overall survival; ORR: overall response rate; mDOR: median duration of response; DCR: disease control rate; OS: overall survival; TriTAC: Tri-specific T Cell Activating Construct; CAR-T: Chimeric Antigen Receptor T-cells; NK: natural killer cells; GD2: disialoganglioside; LAG-3: lymphocyte activation gene 3.

T-cell immunoglobulin and ITIM domain (TIGIT) seemed promising as a target, since anti-TIGIT tiragolumab inhibits T-cells and NK cells. Unfortunately, in Skyscraper02, the median OS of 13.6 months in both arms ended the role of tiragolumab in metastasized SCLC [82]. In the rest of the world, the use of checkpoint inhibitors in SCLC is standard therapy in combination with chemotherapy in first line. Atezolizumab and durvalumab have current FDA approval, achieving a modest though important survival benefit without selection criteria.

Current ongoing trials are evaluating the combination of anti-PD1/anti-PDL1 and anti-TIGIT in LD-SCLC. In this light, the NCT04308785 represents a phase-II study concentrated on the efficacy and safety of atezolizumab, whether or not associated with tiragolumab (anti-TIGIT), as a consolidation therapy in LD-SCLC patients who have not progressed during/after CRT [83], while the NCT04952597 phase-II trial examines the combination of ociperlimab plus tislelizumab plus concomitant CRT [84].

Future strategies involve the DLL3-targeted bispecific T-cell engager (BiTE) tarlatamab, which binds both DLL3 and CD3, leading to tumor lysis [101]. The phase-1 DeLLphi-300 confirmed an ORR of 23.4% and DCR of 51% for tarlatamab in 107 patients with metastasized SCLC [85]. Median OS was 13.2 months, while 36.4% of patients showed a target lesion shrinkage greater than 30%. Of concern is the serious adverse event cytokine release syndrome occurring in cycle 1 and rarely occurring in subsequent cycles. Combination with anti-PD-L1 is thought to overcome resistance in T-cell cold tumors [102]. Tarlatamab, administered as a 10-mg dose every two weeks in patients with previously treated SCLC (DeLLphi-301) showed an ORR of 40% and a DCR of 59%. The median PFS was 4.9 months [86]. Currently, patients are included in DeLLphi-304, phase

3: tarlatamab monotherapy in second line after prior platinum-based therapy [88]. And opening soon: DeLLphi-303: tarlatamab plus standard of care first line [87].

BI764532 is another bispecific T-cell engager against DLL3/CD3 currently being developed and recruiting patients for phase 1 [91].

HPN328 is a novel tri-specific recombinant protein construct (Tri-specific T Cell Activating Construct [TriTAC®]) DLL3-targeting T-cell engager tested in SCLC, but also in other neuroendocrine tumors expressing DLL3 [103]. The first results of 18 patients revealed a decrease in disease in 7 patients (39%). In 11 SCLC patients, 3 patients (27%) achieved a decrease greater than 30% [95]. No dose-limiting toxicity occurred, grade 1 – 2 cytokine release syndrome was reported in 4 patients (22%).

ADC, BiTes and CAR-T for other targets are also being developed, against B7-homolog3 (B7-H3) and disialoganglioside (GD2) [104 - 106].

Another targetable immune checkpoint in SCLC-I includes lymphocyte activation gene-3 (LAG3). LAG3 expression had a trend towards a better OS, and turned out to be related to immune-related processes, such as immune response, antigen processing and presentation, and T-cell co-stimulation [107]. Chimeric antigen receptor therapy (CAR-T) uses the T-cells to recognize cancer cells, a novel application in SCLC.

Another strategy is to activate the Stimulator of Interferon Genes (STING) pathway, which facilitates an immune response via CD8+ cytotoxic T cell infiltration [108]. DNA damage response (DDR) proteins PARP and checkpoint kinase 1 (CHK1) increase the protein and surface expression of PD-L1[108], thus enhancing the effect of immunotherapy. Both DDR inhibition and WEE1 inhibitors activate the STING pathway, and, in combination with chemotherapy or PD-L1 inhibition, cause remarkable tumor regression [109, 110].

Preclinical research of in-vitro profiling of plasticity in SCLC cells revealed upregulation of the major histocompatibility complex class 1 (MHC-1). Transient combined EZH2 inhibition and STING agonism prime cells for immune rejection, promoting durable immunotherapy benefits [111 - 113]. Furthermore, targeted inhibition of lysine-specific demethylase 1 (LSD1) restores the major histocompatibility complex class 1 (MHC-1) and sensitizes SCLC cells to MHC-I restricted T-cell cytolysis [114, 115]. The combination of the LSD1 inhibitor with immunotherapy augments the anti-tumor immune response in refractory SCLC [115]. These findings represent a promising immunotherapeutic approach in SCLC.

We are left with a few unanswered, though relevant, questions: how can we identify patients who will derive a long-term benefit from immunotherapy? What is the role of checkpoint inhibitors in limited stage SCLC? Do patients need ongoing, indefinite therapy to prevent a relapse? Can we target other biomarkers with medication to speed up the trend towards precision medicine and better outcomes for our patients?

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Chapter 4

O^6-Methylguanine-DNA methyltransferase (MGMT): A druggable target in lung cancer?

Birgitta I. Hiddinga
Patrick Pauwels
Annelies Janssens
Jan P. van Meerbeeck

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ABSTRACT

This manuscript addresses the role of O^6-methylguanine-DNA methyltransferase (MGMT) as a biomarker in the oncogenesis of cancer and the opportunity of turning this gene into a druggable target in neuroendocrine tumors of the lung. Studies in brain tumors conclude that MGMT promoter methylation is considered a strong predictive factor for a favorable outcome for treatment with temozolomide, e.g. alkylating agent. We conducted a systematic review of MGMT in non-small cell lung cancer (NSCLC), small-cell lung cancer (SCLC) and other pulmonary neuroendocrine tumors (NETs) to evaluate whether MGMT is a prognostic and/or predictive factor to select patients with lung cancer who can benefit from treatment with temozolomide. In NSCLC MGMT promoter methylation is not a prognostic and predictive factor, hence temozolomide has no place. In SCLC and NET patients with a MGMT promoter methylation benefit of temozolomide has to be confirmed. Temozolomide can be considered a 'personalized' treatment if the predictive role of MGMT is further confirmed.

Highlights

- MGMT promoter methylation predicts response to treatment with alkylating agents in patients with glioblastoma multiforme and possibly in small cell lung cancer and pulmonary neuroendocrine tumors.
- In small cell lung cancer and large cell neuroendocrine carcinoma there's a high unmet need for new treatment options to improve the prognosis. Repurposing of drugs is an acceptable and innovative strategy in the advancement of treatment.
- Temozolomide can be considered a 'personalized' treatment in lung cancer if the predictive role of MGMT is further confirmed.

INTRODUCTION

The treatment of advanced non-small cell lung cancer (NSCLC) has recently seen a paradigm shift with the discovery of several actionable somatic alterations in genes linked to hallmark pathways of cancer as EGFR, ALK, c-MET, ROS. These genomic alterations have led to the development of targeted oral small molecules specifically blocking the activated kinases of these pathways. These small molecules have since been registered for the treatment of a small subset of patients with non-squamous NSCLC with so-called druggable oncogenic mutations, for vastly improving their progression free survival (PFS) compared to standard chemotherapy.

Neuroendocrine tumors (NETs) of the lung are a morphologically and clinically distinct subgroup representing less than 20% of lung cancers and encompassing a spectrum from the more benign carcinoid (grade 1) and atypical carcinoid (grade 2) tumors, to the highly aggressive neuroendocrine carcinomas (NECs) grade 3 and 4: small cell lung cancer (SCLC) and large cell neuroendocrine (LCNEC) variants with a high metastatic potential and a poor prognosis [1]. Their common phenotypic characteristic is the expression of features as neuroendocrine granules and the secretion of paraneoplastic cytokines and hormones, which reflect a common origin from the embryonal neuroendocrine crest. NETs arise from cells throughout the endocrine system. Although the different types of pulmonary NETs originate from the Kulchitsky cells of the bronchial mucosa, different mutations cause different biology and they are therefore considered separate clinical entities [2]. Most NETs are sporadic, and risk factors are poorly understood. Smoking may be an important risk factor for their development, especially in atypical carcinoids and the NECs SCLC and LCNEC [3]. Pulmonary LCNEC is recognized as a variant of NSCLC in which further studies are needed to confirm whether a treatment with platinum etoposide, similar as in SCLC, is more appropriate than a combination of platinum with a third generation drug as in NSCLC [4,5].

At the molecular level, SCLC often harbors alterations in the MYC gene family members of transcription factors, amplifications of fibroblast growth factor 1 (FGFR1), silencing of the tumor suppressor *Rb* gene, mutation of the *TP53* gene mutations or an aberrant expression of genes implicated in DNA damage repair, among which the O^6-methylguanine methyltransferase (*MGMT*) gene, responsible for the repair of DNA damage [6]. This manuscript reviews the role of MGMT in the oncogenesis of cancer and the opportunity of turning this gene into a druggable target in tumors of the lung, more specifically for the alkylating agent temozolomide.

The search for prospective and retrospective publications relative to lung cancer, MGMT and temozolomide was performed by consulting PubMed. Search terms used included “MGMT”, “MGMT promoter methylation”, “MGMT promoter hypermethylation”, “MGMT expression” in combination with MeSH key words as “temozolomide” and “lung cancer”; “NSCLC”; “SCLC”; “neuroendocrine tumor”; “NET” and “NEC”. PubMed was searched for relevant articles; phase I; II and III; prospective and retrospective between 2002 and 2014. In addition; the retrieved articles were searched for cross references. We retrieved a total of 40 publications that are listed in Table 1.

WHAT IS THE ROLE OF MGMT IN ONCOGENESIS?

Cells deploy a wide variety of enzymes to accomplish the very challenging task of continuously monitoring the integrity of the genome and to remove inappropriate base or nucleotides created by chemical or physical attacks, and to replace them with those bases or nucleotides that existed prior to the attack. The simplest strategy for restoring the structure of chemically altered DNA involves an enzyme-catalyzed reversal of the chemical reaction that initially created the altered base. As such, a DNA-alkyltransferase can remove methyl and ethyl adducts from the O^6 position of guanine, thereby restoring the structure of the normal base. The importance of this enzyme, MGMT, as a DNA alkyltransferase in the development of certain kinds of human tumors is suggested by observations that the *MGMT* gene is silenced by promoter methylation in as many of 40% of gliomas and colorectal tumors, and in about 25% of NSCLC’s, lymphomas and head and neck cancers [7].

DNA-promoter methylation is a well-known epigenetic process and refers to binding of a methyl group to cytosine nucleotides in the DNA sequence. Aberrant promoter methylation of CpG islands in the promoter regions of tumor cells is one of the major mechanisms for silencing of tumor suppressor genes. The DNA repair protein encoded by the *MGMT* suppressor gene removes alkyl groups from the O6 position of guanine [8]. The epigenetic silencing of the *MGMT* gene via promoter methylation of specific CpG islands of its promoter, hence leads to loss of expression of MGMT enzyme [9].

In tissue, MGMT promoter methylation status can be assessed by polymerase chain reaction (PCR) and MGMT expression by immunohistochemistry (IHC). This can be adequately done on cytology or small biopsies [10]. Quality and validation of the tests are important for an accurate assessment [11,12]. However, MGMT quantification by IHC varies greatly between laboratories because of the difficulty of the method [13] . Expression of MGMT proteins varies, with lower MGMT levels in tumor tissue than

in normal tissue. There seems no clear correlation between the expression of MGMT protein in tumor compared to the MGMT promoter methylation.

WHAT IS THE PROGNOSTIC ROLE OF MGMT IN NSCLC?

MGMT promoter methylation status in NSCLC was assessed in a review of 18 studies [14]. The presence of MGMT promoter methylation was tested in 1160 tumor samples and in 970 control samples of either plasma, serum or bronchoalveolar lavage fluid. The odds ratio of MGMT promoter methylation was higher in tumor tissue than in control samples (OR = 4.43, 95% CI: 2.85–6.89), suggesting that *MGMT* gene promoter methylation is frequent in NSCLC. Profiles are different in adenocarcinoma and squamous cell carcinoma [15].

In a prospective series of patients with NSCLC, MGMT promoter methylation determined by PCR was observed in patients with NSCLC, but not in the mucosa of healthy controls [16]. The promoter methylation frequency ranged from 0 to 50%, with an overall index of 18%. Even in sputum of cancer-free smokers, MGMT promoter methylation is detectable, suggestive of a correlation with the risk of lung cancer [13,17]. Similarly, a low protein expression of MGMT was found in bronchial epithelium of patients with lung cancer, compared to healthy controls, suggesting that there is an association between MGMT expression and lung cancer risk [18]. Further validation whether MGMT-expression is a marker for early detection of lung cancer is needed [19]. Histologically negative bronchial margins of resected lung tumors frequently exhibit promoter methylation changes. These may represent a field of preneoplastic changes for recurrence of NSCLC [20]. In comparing the promoter methylation patterns of malignant and non-malignant lung tissues from the same patients, many discordances occurred whereby the genes methylated in non-malignant tissues are not methylated in the matching tumor tissues. This suggests that promoter methylation is possibly a preneoplastic change.

The prognostic role of MGMT promoter methylation was tested in three surgical series. These studies reported that 15–51% of patients tested positive for MGMT promoter methylation. Brabender et al. reported the promoter methylation status of the *MGMT* gene in 34 of 90 (38%) resected NSCLC samples and 16 of 90 (18%) matching normal tissue [21]. MGMT promoter methylation in normal tissue was always accompanied by MGMT promoter methylation in matched tumor tissue. Patients without MGMT promoter methylation showed a significantly better survival than patients with the promoter methylation, suggesting that MGMT promoter methylation is a prognostic biomarker for a more aggressive behavior of NSCLC. This is in contrast with patients with

glioblastoma multiforme (GBM), in whom MGMT promoter methylation is considered a favorable prognostic variable [22].

In a prospective surgical series in patients with stages pI to pIIIA, MGMT promoter methylation was present in 8% of the cases and was not associated with improved overall survival (OS) or recurrence-free survival (RFS) [23]. In a retrospective analysis of resected NSCLC specimens, reduced or absent MGMT protein expression was found in 48 of 112 NSCLC's (43%), and was significantly correlated with nodal metastasis and squamous or undifferentiated cell types and p53 overexpression, suggesting that loss of MGMT expression plays a role in disease progression [24].

Retrospective analysis of MGMT expression and survival of 108 patients with stage I and II NSCLC led to the conclusion that there was no correlation [25]. MGMT expression was reduced in 18.1% of poorly differentiated tumors and in 77.8% of large cell carcinoma cases. MGMT promoter methylation however, was associated with a poor prognosis. Other studies found no relationship between MGMT promoter methylation and survival in resected patients [26].

In conclusion, MGMT promoter hypermethylation in lung cancer is frequent, but the prognostic association is unclear and limited to the earlier stages of NSCLC.

WHAT IS THE PROGNOSTIC ROLE OF MGMT IN BRAIN METASTASES OF LUNG CANCER?

Brain metastases are a common complication of patients with lung cancer, occurring in approximately 25% of patients. Brain metastases have a major impact on quality of life with a poor median survival of around 4 months. As promoter methylation of the *MGMT* gene is frequently observed in GBM, a retrospective analysis assessed MGMT expression with IHC and promoter methylation with PCR in 86 biopsies of brain metastases of lung cancer [27, 28]. Paired specimens of lung were available for 20 patients. MGMT protein expression was seen in 71 of 86 (83%) brain metastases and 10 of 20 (50%) primary lung cancers ($p = 0.004$). There was a trend toward lower frequency of MGMT protein expression in SCLC compared to NSCLC (50% versus 85%, $p = 0.063$). No correlation was found between the MGMT protein expression or promoter methylation status in brain metastases and paired lung tumor tissue. All tumors with MGMT promoter methylation were negative for MGMT protein expression, and tumors with protein expression did not have the MGMT promoter methylation. Furthermore, no correlation was found between MGMT promoter methylation status or expression and response to prior chemotherapy. MGMT promoter methylation in brain metastases was inversely correlated with survival.

Patients with MGMT protein expression in brain metastases had significantly longer median survival than those without (16.5 versus 3.5 months, $p < 0.001$). MGMT protein expression in brain metastases was hence considered an independent prognostic factor.

MGMT promoter methylation assessment in brain metastases of patients with NSCLC treated by resection followed by whole brain radiotherapy (WBRT), showed that the relapse rate was higher in methylated tumors [29] . In this retrospective series, 55 patients were included of which only 5 patients (9.1%) had a methylated *MGMT* gene. The median PFS in the brain was 4.0 months with and 11.5 months without promoter methylation. The median overall survival time (MST) was 6.2 months with promoter methylation and 20.9 months without promoter methylation. The promoter methylation status was not correlated to any patient characteristics. Due to an insufficient number of patients with MGMT promoter methylation, MGMT promoter methylation status was not prognostic for the outcome.

In conclusion, the findings in brain metastases of lung cancer corroborate the ones described earlier in NSCLC.

WHAT IS THE PROGNOSTIC ROLE OF MGMT IN NETS?

In retrospective series, MGMT is hypermethylated in 27% of lung NETs [30], and 40% of pancreatic NETs [31]. Loss of MGMT protein was associated with adverse outcomes in a surgical series, but not independently from stage and grade of the disease. No prospective data are available in lung NETs. The profile of MGMT promoter methylation and protein expression in NETs is different from that of NSCLC [15]. The overall pattern of promoter methylation in SCLC and carcinoids are comparable, carcinoids having lower frequencies of promoter methylation than SCLC.

WHAT IS THE RELATIONSHIP BETWEEN MGMT AND ALKYLATING AGENTS?

Alkylating agents leads to cell death by alkylation of the O6- position of guanine and subsequent disturbance of DNA replication [32]. Presence of a non-silenced *MGMT* gene is considered predictive of resistance to alkylating agents [33–36]. By promoter methylation, the *MGMT* gene is silenced, the cell becomes unable to repair its damaged DNA. Unrepaired DNA lesions trigger apoptosis and hence an increased sensitivity to DNA damaging agents, e.g. alkylating drugs. Low MGMT protein expression has been associated with response to alkylating drugs in GBM, which is an aggressive form of brain cancer with a median survival of less than a year [37].

Temozolomide (Temodal™)is a triazene derivative, adding an alkylating group on DNA [38]. It is a prodrug and has its activity after conversion into monomethyl triazenoimidazole carboxamide (MTIC). Oral absorption of the drug is rapid [32]. Temozolomide is well-tolerated and toxicities are mild [39]. Trombocytopenia is the most common adverse event, causing dose modification or treatment discontinuation. Grade 4 toxic effects are rare. No consensus is achieved on the optimal dosage of temozolomide. Protracted schedules of temozolomide may lead to reduction of the cell's capability for MGMT-mediated DNA repair and resistance by the prolonged depletion of MGMT activity [40]. A randomized phase III trial of adjuvant dose-dense temozolomide (75 mg/m2 day 1–21 of a 28-day cycle) versus standard temozolomide (75 mg/m2 day 1–5 of a 28-day cycle)in newly diagnosed GBM did not improve efficacy, regardless of MGMT promoter methylation [41]. Temozolomide crosses the blood-brain barrier and patients with brain metastases might benefit from this treatment [42]. The drug was extensively studied in brain tumors, more specifically in GBM [37, 43]. Based on these results, temozolomide is approved for use in patients with GBM and refractory high grade astrocytoma [44] . Several studies conclude that the benefit of temozolomide is largest in tumors that have a MGMT promoter methylation, and are thus unable to repair the chemotherapy-induced DNA damage [33, 45, 36]. The epigenetic silencing of the DNA-repair enzyme MGMT is hence considered a strong predictive factor for favorable outcome in patients with GBM treated with temozolomide [46].

Little is known about the resistance mechanisms to temozolomide. Resistance to temozolomide is likely predicted by the presence of a somatic *MSH6* mutation independently of MGMT promotor promoter methylation [47,48]. Another mechanism of resistance is caused by alkylpurine-DNA-N-glycosylase (APNG), also known as DNA methylpurine-N-glycosylase (MPG)[49]. Evaluation of APNG protein levels demonstrated that high nuclear APNG expression was correlated with a poorer overall survival in patients with GBM. An important role in tumor resistance to alkylating agents is the potent MGMT-inactivating agent O6-benzylguanine (O6-BG). Preclinical data suggested that the combination of lomeguatrib (O6-(4-bromothenyl)guanine) and temozolomide can overcome the resistance mechanism and improve inhibition of tumor growth [45,50]. A new drug to overcome resistance may be NE0212, a temozolomide analog [51].

WHAT IS THE ACTIVITY OF TEMOZOLOMIDE IN NSCLC?

As temozolomide crosses the blood-brain barrier with therapeutic concentrations to the brain and in view of its activity in primary brain tumors, the drug was extensively tested in the prevention and treatment of brain metastasized lung cancer [52]. Temozolomide was tested in first line [53], as maintenance [54] and in pretreated patients [55–61]. As

temozolomide has a radiosensitizing effect [62], combinations of temozolomide with WBRT were conducted [63–71] (Table 1).

The EORTC LCG 08965 performed a phase II study with temozolomide in chemo-naïve patients with advanced NSCLC and compared 12 patients with and 13 patients without brain metastases [53]. Patients received 200 mg/m2 for 5 days of a 28-day cycle. First-line treatment with temozolomide did not demonstrate any activity in these stage IV NSCLC. Investigator bias can have resulted in a patient group with a relatively poor performance status, since a platinum-doublet was considered too challenging in these patients. Temozolomide maintenance after completion of first-line therapy for NSCLC also does not decrease the incidence of brain metastases in patients with locally advanced NSCLC [54]. Temozolomide has hence no place in the first line or maintenance treatment of patients with NSCLC with or without brain metastases.

Temozolomide has been studied in pretreated patients with advanced NSCLC (Table 1) [55–60]. Its activity and survival times are comparable to other drugs in the second line treatment of advanced NSCLC. Temozolomide was well tolerated and may be a reasonable treatment option for patients with brain metastases of lung cancer.

Only one phase 2 study with temozolomide investigated whether MGMT promoter methylation was linked to the response to temozolomide [61]. Of 740 patients with a solid tumor, 86 showed MGMT promoter methylation, of which 13 of 242 for NSCLC (5%). The response rate (RR) for the patients with NSCLC was 0%. The efficacy of temozolomide in patients with NSCLC with confirmed methylated MGMT status is nihil.

As temozolomide has a radiosensitizing effect, the combination with WBRT may improve its therapeutic effect [62] . Studies were conducted in patients with brain metastases and the combination seems safe and feasible (Table 1). However, the benefit of adding temozolomide to WBRT in NSCLC is not confirmed. One trial showed no survival improvement, but statistically significant improved response rates with addition of temozolomide to WBRT [63]. Results are difficult to interpret in a patient series in which extra-cranial progression had a considerable impact on prognosis. The efficacy of concomitant temozolomide on survival remains hence unclear [64]. In these studies, no MGMT biomarker assessment was done [65–71].

Combinations of temozolomide and other cytotoxic agents have been tested in an attempt to improve efficacy [72–84] (Table 1). Vinorelbine has a broad-spectrum in vivo and in vitro action in solid tumors and may enhance penetration across the blood-brain barrier [73]. A phase I study the combination of vinorelbine and temozolomide

Table 1. Temozolomide in lung cancer

Temozolomide in NSCLC					
Author [reference]	**Design**	**Patients**	**N**	**biomarker**	**brain-metastases**
Dziadzuiszko [53]	phase 2, single arm temozolomide	first-line	25	no	12 (48%)
Kouroussis [55]	phase 2, single arm temozolomide	pretreated patients	31	no	12 (39%)
Giorgio [56]	phase 2, temozolomide	≥ second-line	30	no	100%
Adonizio [57]	phase 2, single arm temozolomide	≥ second-line	38	no	
Abrey [58]	phase 2, single arm temozolomide	≥ second-line	22 NSCLC, 2 SCLC	no	100%
Siena [59]	phase 2, single arm, two step temozolomide	pretreated	53 (34%)	no	100%
Christodoulou [60]	phase 2, single arm temozolomide	pretreated	12 NSCLC, 5 SCLC of 28 pts	no	100%
Hochhauser [61]	phase 2, single arm temozolomide	pretreated	242	MGMT promoter methylation	
Antonadou [63]	phase 2, randomized, WBRT +/- temozolomide	pretreated		no	100%
Minniti [64]	temozolomide + second course of WBRT	pretreated	18 (of 27)	no	100%
Chua [65]	phase 2, randomized, WBRT +/- temozolomide	pretreated	47	no	100%
Mikkelsen [66]	phase 1 / 2 dose escalation temozolomide + WBRT	pretreated	13	no	100%
Addeo [67]	phase 2, temozolomide + WBRT	pretreated	27	no	100%
Wang [68]	retrospective, temozolomide + WBRT	pretreated	32	no	100%
Hassler [69]	phase 2, temozolomide + WBRT, closed due to poor accrual	pretreated		no	100%
Kouvaris [70]	phase 2, temozolomide + WBRT	pretreated	11 (of 33)	no	100%
Verger [71]	phase 2, randomized, WBRT +/- temozolomide, closed due to poor accrual	pretreated		no	100%
Omuro [74]	phase 1, single arm, temozolomide + vinorelbine	pretreated		no	
Iwamoto [75]	phase 2, single arm, temozolomide + vinorelbin	pretreated	17 NSCLC and 3 SCLC	no	100%
Tamaskar [77]	phase 1, single arm, temozolomide + docetaxel	pretreated		no	
Caraglia [78]	phase 2, single arm temozolomide + pegylated liposomal doxorubicin	pretreated	6 (31.6)	no	100%
Choong [79]	phase 2, randomized, temozolomide vs irinotecan	second-line	46	no	
Britten [80]	phase 1, single arm, temozolomide + cisplatin	pretreated		no	
Christodoulou [81]	phase 2, single arm, temozolomide + cisplatin	pretreated	32 (lung and breast)	no	

Dosage temozolomide	Response	Median TTP (mo)	MST (mo)	1 year survival rate (%)	Toxicity
200 mg/m² day 1 - 5 of a 28 day cycle	no responses				
75 mg/m² day 1 - 21 of a 28 day cycle	2 PR, 3 SD	2.4	3.3	22.5	1 grade 5 neutropenia
150 mg/m² day 1 - 5 of a 28 day cycle	2 CR, 1 PR, 3 SD	19 mo in patients with CR, 11 months in patients with PR			no grade 3 / 4
75 mg/m²/day for 6 weeks of 8 to 10 weeks	1 CR, 2 PR, 12 SD, 19 PD				no grade 4 / 5
150 mg/m² day 1 - 5 of a 28 day cycle	DCR 40%				no grade 4 / 5
150 mg/m² day 1 - 7 and day 15 - 21 of a 28 day cycle	2% CR, 4% PR, 21% SD, DCR was 29% with WBRT and 18% without WBRT	2.2	5.7		mild, mostly trombocytopenia
150 mg/m² day 1 - 5 of a 28 day cycle	1 PR in NSCLC, 4 SD (17%)				no grade 3 / 4
150 mg/m² day 1 - 7 and day 15 - 21 of a 28 day cycle	4 SD (40%), 5 PD (50%), 1 NA, RR 10% in NSCLC				
75 mg/m² day during radiotherapy, thereafter 200 mg/m² day 1 - 5 of a 28 day cycle, for 6 cycles					
75 mg/m² day 1 - 10					
75 mg/m² day 1 - 28 of a 35 day cycle		3.1	4.4		mild to moderate, 6% ≥ grade 3
MTD 95 mg/m2 day during WBRT	3 PR (18%), 10 SD (59%)	2.4			
75 mg/m² day 1 - 10, followed by 75 mg/m² day 1 - 21 of a 28 day cycle					15% grade 3 neutropenia, 13% grade 3 neutropenia, 1 patient with grade 4 trombocytopenia
	1 CR, 9 PR, 15 SD	5.5	8		no grade 3 / 4
75 mg/m² day during radiotherapy, thereafter 200 mg/m² day 1 - 5 of a 28 day cycle, for 6 cycles	RR 54.5%, RR in brain 78.6%		12		
75 mg/m² day during radiotherapy, thereafter 200 mg/m² day 1 - 5 of a 28 day cycle, for 2 cycles					
150 mg/m2 day 1 - 7 and day 15 - 21 of a 28 day cycle	1 CR in NSCLC, 5 SD, 29 PD		5 (in the patient with CR)		
200 mg/m2 day 1 - 5 of a 28 day cycle	7 PR (37%) in 19 patients (all solid tumours)				no grade 4 / 5
	4 PR, 17 SD	1.8	9.8	34	9% grade 3 / 4 leucopenia and diarrhea, 1 grade 5
150 - 200 mg/m2 day 1 - 5 of a 28 day cycle	10 PR (31%), 5 SD (16%)				grade 3 / 4 mostly hematological, 1 grade 5 neutropenic fever

Table 1. Continued

Temozolomide in NSCLC					
Cortot [82]	phase 2, temozolomide / cisplatin + WBRT			no	
Sperduto [83]	phase 3, randomized, WBRT + SRS or WBRT + SRS + temozolomide or WBRT + SRS + erlotinib			no	100%
Pesce [84]	phase 2, randomized, WBRT + gefitinib or WBRT + temozolomide	pretreated		no	100%
Temozolomide in SCLC					
Author [reference]	**Design**	**Patients**	**N (number)**	**biomarker**	**brainmetastases**
Pietanza [86]	phase 2, single arm, temozolomide	≥ second-line		MGMT promoter methylation	
Zauderer [87]	phase 2, single arm, temozolomide	pretreated	25	MGMT protein expression and MGMT promoter methylation	
Temozolomide in NETs					
Author [reference]	**Design**	**Patients**	**N**	**biomarker**	
Chong [94]	retrospective, temozolomide-based	pretreated	14	no	
Ekeblad [95]	retrospective, single arm temozolomide	pretreated	13	MGMT protein expression	
Crona [96]	retrospective, single arm temozolomide	pretreated	31	no	
Kulke [100]	phase 2, prospective, single arm temozolomide + bevacizumab	pretreated		no	
Koumarianou [101]	phase 2, prospective, single arm temozolomide + bevacizumab + octreotide	pretreated		no	
Chan [102]	phase 2, prospective, single arm temozolomide + bevacizumab		4 (12%)	no	
Kulke [103]	phase 2, prospective, single arm temozolomide + thalidomide	pretreated	15	no	
Isacoff [104]	retrospective, temozolomide + capecitabine				
Spada [105]	retrospective, temozolomide + capecitabine			no	
Strosberg [106]	retrospective, temozolomide + capecitabine			Ki-67	
D'Alpino Peixoto [107]	temozolomide + capecitabine		3 (10.9%)	no	

NSCLC: non-small cell lung cancer; SCLC: small-cell lung cancer; NETs: neuroendocrine tumors; TTP: time to progression; MST: median survival time; MTD: maximum tolerated dose; WBRT: whole brain radiotherapy; SRS: stereotactic radiosurgery; RR: response rate; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; DCR: disease control rate; NS: not stated

					grade 3 / 4 neutropenia (20%) and trombopenia (22%)
			4.9		fatigue

Dosage temozolomide	Response	Median TTP (mo)	MST (mo)	1 year survival rate (%)	Toxicity
75 mg/m2 day 1 - 21 of a 28 day cycle	RR 22% in the unselected group, RR 38% in patients with brain metastases, RR 19% in third line				
200 mg/m2 day 1 - 5 of a 28 day cycle	PR in 12 pts (48%), no responses in the brain				grade 3 / 4 hematologic in 5 patients (20%)

Dosage temozolomide	Response	Median TTP (mo)	MST (mo)	2 year survival rate (%)	Toxicity
	RR 14% (2 pts), 57% DCR (8 pts)	10			
200 mg/m2 day 1 - 5 of a 28 day cycle	0 CR, 31% PR (4 pts), 31% SD (4 pts), 38% PD (5 pts)	7			grade 3 / 4 hematologic toxicity in 4 of 35 pts
	14% PR, 52% SD	5.3	23.2		
150 mg/m2 day 1 - 7 and day 15 - 21 of a 28 day cycle	92% SD				10% infection during lymphopenia
	1 pt with PR			70%	
200 mg/m2 day 1 - 14 of a 28 day cycle					
200 mg/m2 day 1 - 14 of a 28 day cycle					
200 mg/m2 day 1 - 14 of a 28 day cycle					
200 mg/m2 day 1 - 14 of a 28 day cycle					

was given to patients with brain metastases of solid tumors and this combination is safe and feasible [74]. However, the phase II trial of the combination vinorelbine and temozolomide did not improve response rates compared to previous studies with single-agent temozolomide. In this group of patients to receive a treatment with palliative intent, the high observed incidence of toxicity was unacceptable [75]. Synergism of temozolomide was found with gemcitabine and paclitaxel, but not with platinum analogs and topoisomerase inhibitors [76]. Although the combination of docetaxel, doxorubicine, irinotecan or cisplatin with temozolomide was safe and feasible for patients with brain metastases, no objective responses were seen [77–81]. Association of cisplatin to temozolomide followed by WBRT also demonstrated a lack of efficacy and was considered too toxic with a grade 3 and 4 neutropenia and trombopenia occurring in 20% and 22% of patients, respectively [82]. The addition of erlotinib or gefitinib didn't improve survival and had a possible deleterious effect [83,84].

The principal limitation of the studies with temozolomide in brain metastases is the inclusion of different solid tumor types. The activity of monotherapy temozolomide is not proven in patients with NSCLC. In combination with WBRT its activity has to be confirmed. With a small number of studies that used MGMT promoter methylation as a biomarker, no conclusions can be drawn whether to predict a response on treatment with temozolomide in NSCLC.

WHAT IS THE ACTIVITY OF TEMOZOLOMIDE IN SCLC AND NETS?

Anecdotal responses to temozolomide have been noted in SCLC [85]. Beneficial effects have been reported, especially in a subgroup associated with the presence of MGMT promoter methylation, although the comparison did not meet statistical significance and was analyzed retrospectively [86]. Patients with progressive SCLC after one or two prior chemotherapy regimens received temozolomide at 75 mg/m2 daily for 21 days of a 28-day cycle. The primary endpoint was RR. The RR of 22% is in an unselected group and even in third line the RR was 19%. In the group SCLC with brain metastases the RR was 38% (Table 1).

Twenty-five patients were enrolled in a single center trial of a 5- day dosing regimen of temozolomide 200 mg/m2 in a 28-day cycle [87]. The rationale for this shortened dosing schedule was to avoid prolonged myelosuppression. The primary endpoint, tolerability, was met with grade 3 and 4 toxicity in only 5 patients. Temozolomide was well-tolerated. Responses were seen in 12 patients (48%, 95% CI: 3–31%). No responses in the brain were seen with this regimen. Assessment of MGMT expression with IHC was

performed in 52% (13 of 25) of patients on archival tissue. One (8%) of the MGMT IHC was negative and achieved a partial response. Eight tissues were tested for the MGMT promoter methylation and of these, 7 had evidence of promoter methylation of whom 1 had a partial response. The small sample size does not allow to draw solid conclusions about the predictive value of MGMT silencing. A study in relapsed or refractory SCLC is recruiting patients with MGMT promoter methylation to be treated with temozolomide 200 mg/m2 for 5 days of a 28 day cycle until progression or intolerability [88].

Preclinical data show that veliparib, a potent oral inhibitor of poly(ADP-ribose) polymerase (PARP) enhances the activity of temozolomide [89]. An ongoing phase II trial is comparing the PARP-inhibitor veliparib with temozolomide or temozolomide alone in patients with relapsed SCLC [90].

Recent guidelines recommend temozolomide treatment in advanced unresectable progressive pulmonary atypical carcinoid tumors [91]. However, no agreement was achieved on its optimal dosing regimen and schedule [92]. Furthermore, the NET task force recommended that carcinoid tumors and pancreatic NETs should be evaluated separately or stratified by primary site in large randomized trials [93]. All publications were prospective or retrospective single arm studies [94–107]. In a retrospective series of 300 patients with pulmonary carcinoid (80 patients with AC), 14 patients were treated with a temozolomide-based therapy [94](Table 1). A RR of 14% (2 of 14 patients) was seen, with 57% disease control rate (DCR) (8 of 14 patients) and a median PFS of 10 months. In another retrospective series of advanced NETs of which 13 pulmonary, 36 patients were treated with monotherapy temozolomide 200 mg/m2 for 5 days of a 28-day cycle [95]. In the pulmonary carcinoids no complete responses were noted. In 4 patients a partial response (31%) was achieved, and stable disease in 4 (31%) and progressive disease in 5 (38%) patients. Toxicity was mild, there were no fatal side effects. However, dose reduction was necessary due to hematologic reasons in 4 of 35 patients evaluable for toxicity. Survival data for the bronchial tumors was not separately mentioned, but the median time to progression for the whole treatment group was 7 months (95% CI: 3–10). There was no significant difference in time to progression between patients with bronchial tumors and the other organ sites. The MGMT protein expression was assessed in 23 patients. There was no significant difference in the RR between tumors with low or high MGMT expression.

The efficacy of temozolomide was retrospectively analyzed in 31 patients with progressive metastatic pulmonary carcinoids (14 typical, 15 atypical, 2 not classified) [96]. Fourteen percent achieved a partial response and 52% a stable disease with a median PFS of 5.3 months and a median OS of 23.2 months.

Some mixed series of NETs contain only a few patients with pulmonary NETs [97]. The median OS and PFS showed a trend towards a better survival for the pancreatic NETs compared to the non-pancreatic NETs. The first report on second and third line temozolomide-based chemotherapy with NETs after progression, showed a RR of 33% and a DCR of 38% [98].

Temozolomide has been used in monotherapy or in combination with bevacizumab or capecitabine. The rationale for adding an angiogenesis inhibitor to the treatment is that NETs are characterized by abundant vasculature and high levels of vascular endothelial growth factor (VEGF) expression, and therefore possibly susceptible for targeted agents involving angiogenesis [99]. A phase II study of the combination temozolomide and bevacizumab showed an objective response in 24%ofthe patients with pancreatic NET's, while 70% had stable disease [100]. No radiologic responses were seen in the patients with carcinoid, but 92% showed stable disease. Other prospective trials combining temozolomide and bevacizumab confirm the RR of 18% in pancreatic NETs, although the numbers of treated patients with lung NETs are too low to draw any valid conclusions, there is a trend towards better response in pancreatic NETs than lung NETs [101,102].

The antiangiogenetic drug thalidomide was evaluated in combination with temozolomide in a phase II study of patients with NETs [103]. Of 29 patients, 15 had a carcinoid. The group reached an overall objective radiologic response rate of 25% and a 2-year survival rate of 70%. Fourteen patients with carcinoid were evaluable for response. In only 1 patient a radiologic partial response was seen.

The combination of capecitabine and temozolomide for pulmonary NETs has not prospectively been investigated. Retrospective data present a better outcome in patients with pancreatic NETs than in pulmonary NETs treated with capecitabine 1500 mg/m2 on day 1–14 and temozolomide 200 mg/m2 on days 1–14 of a 28-day cycle [104–107] . There is a trend towards a better RR in patients with a proliferation marker Ki–67 > 5%, with a more likely response to capecitabine plus temozolomide in pancreatic NETs [108]. With the introduction of everolimus and long-acting octreotide in the treatment of NETs, the role of temozolomide is possibly limited [109,110].

In conclusion, patients with SCLC benefit from temozolomide if MGMT promoter methylation is present, but this has to be confirmed. The presence of MGMT promoter methylation is significantly associated with response to temozolomide in NETs [111]. Pulmonary NETs showed MGMT expression, but no treatment responses were observed [112]. Second line treatment with temozolomide alone or in combination with capecitabine or bevacizumab results in objective responses or stabilization in NET.

Single agent temozolomide or in combination treatment is also associated with better results in digestive NETs than in pulmonary NETs [95].

SUMMARY AND DISCUSSION

Epigenetic alterations in cancer are a potential source of predictive therapeutic biomarkers for personalized cancer treatment. MGMT promoter methylation is the best known example for predicting response to treatment with alkylating agents in patients with GBM. Other epigenetic changes that play a fundamental role in cancer development may have an impact on clinical practice. MGMT messenger RNA (mRNA) is a potential prognostic and predictive factor for the response on temozolomide. Patients with fewer copies of mRNA in their GBM had a longer PFS and OS if treated with temozolomide [113].

We propose to treat patients with refractory or relapsed SCLC and pulmonary NET and MGMT promoter methylation with temozolomide in a prospective phase 2 biomarker enriched trial. If this trial shows the expected improved activity and toxicity, an ensuing randomized phase 2–3 might be considered with overall survival as primary endpoint. This trial might lead to a new standard of care in patients with refractory or relapsing SCLC, where topotecan is currently the only approved treatment. This medication is toxic, with a response rate of 6–17%. There's an unmet need for new treatment options to improve prognosis without adding too much toxicity. In the absence of druggable driver mutations, it is ethical to include patients with relapsed or refractory SCLC in clinical trials. Repurposing of drugs used for other indications and tumor types is an acceptable and innovative strategy in the advancement of treatment of SCLC [114].

CONCLUSION

MGMT gene silencing by promoter methylation is a frequent process in lung cancer, but the prognostic association is unclear and maybe restricted to the earlier stages of NSCLC. Epigenetic silencing of the DNA-repair enzyme MGMT is a strong predictive factor for a favorable outcome in patients with GBM treated with temozolomide. However, in the first-line or maintenance treatment in NSCLC with or without brain metastases, temozolomide has no place. Patients with SCLC benefit from temozolomide if MGMT promoter methylation is present, but this has to be confirmed. The presence of MGMT promoter methylation is significantly associated with response to temozolomide in NETs. Pulmonary NETs showed MGMT expression, but no treatment responses were observed. Temozolomide can be considered a 'personalized' treatment if the predictive role of MGMT is further confirmed.

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Chapter 5

Are anaplastic lymphoma kinase (ALK) and O^6-methylguanineDNA methyltransferase (MGMT) promoter methylation driver biomarkers of pulmonary neuroendocrine tumors (NETs) and carcinomas (NECs)?

Birgitta Hiddinga
Karen Zwaenepoel
Annelies Janssens
Jan van Meerbeeck
Patrick Pauwels

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ABSTRACT

Background

Novel targets in neuroendocrine tumors (NETs) and neuroendocrine carcinomas (NECs) are needed to improve outcome. The presence of O^6- Methylguanine-DNA methyltransferase (MGMT) promoter methylation in NETs and NECs may act as a predictive marker for response on treatment with temozolomide. As anaplastic lymphoma kinase (ALK) plays an important role in the nervous system we hypothesized that ALK rearrangement can act as a biomarker in patients with NETs and NECs.

Materials and Methods

We performed a retrospective analysis to establish the frequency of MGMT promoter methylation and ALK expression in tissue samples of patients with NETs and NECs. Results: 21% (14/67) of patients tested positive for MGMT promoter methylation. MGMT promoter methylation was present in 33% (3/9) patients with typical carcinoid, in 22% (2/9) patients with atypical carcinoid, in 22% (8/37) patients with small cell lung cancer and in 8% (1/12) patient with large cell neuroendocrine carcinoma. ALK- expression was present in 14% (10 of 70 patients). In all of these patients, no ALK-rearrangement nor ALK-mutation was revealed.

Conclusions

Routine testing of NET and NEC samples for an ALK rearrangement is not recommended as ALK-expression is not associated with an ALK-rearrangement. Routine testing of NET and NEC samples for MGMT will detect a promoter hypermethylation in a sizable minority of patients who are eligible for a targeted treatment with temozolomide.

INTRODUCTION

Neuroendocrine tumors (NETs) and neuroendocrine carcinomas (NECs) are a subgroup representing less than 20% of lung tumors, with pulmonary NETs considered an orphan disease with an incidence of about 2% of all lung tumors [1–3]. NETs and NECs encompassing a morphologically and clinically distinct spectrum from typical carcinoid (TC) (grade 1) and atypical carcinoid (AC) (grade 2) tumors, to the highly aggressive neuroendocrine carcinomas (NECs) grade 3 and 4, small cell lung cancer (SCLC) and large cell neuroendocrine (LCNEC) variants with a high metastatic potential and a poor prognosis [4]. Their common phenotypic characteristic is the expression of features as neuroendocrine granules and the secretion of paraneoplastic cytokines and hormones, which reflect a common origin from the embryonal neuroendocrine crest. NETs arise from cells throughout the endocrine system. Although the different types of pulmonary NETs originate from the Kulchitsky cells of the bronchial mucosa, different mutations cause different biology and they are therefore considered separate clinical entities [5].

In the treatment of patients with advanced non-small cell lung cancer (NSCLC), a paradigm shift occurred over the last years by the discovery of actionable driver mutations and translocations susceptible for targeted treatment. Despite extensive research, few innovations in the treatment of NETs and NECs have been proposed. New potential targets in NETs and NECs are needed to improve outcome.

In NETs, DNA-promoter methylation might be a mechanism that maintains the neuroendocrine biology [6]. DNA-promoter methylation is a well-known epigenetic process and refers to one of the major mechanisms for silencing tumor suppressor genes. The DNA repair protein encoded by the O^6-Methylguanine-DNA methyltransferase (*MGMT*) suppressor gene removes alkyl groups from the O^6 position of guanine [7]. The epigenetic silencing of the *MGMT* gene via promoter methylation of specific CpG islands of its promoter leads to loss of expression of MGMT enzyme [8]. MGMT promoter methylation status can be assessed by polymerase chain reaction (PCR) on either a cytology specimen from (needle) aspirations or a tissue specimen from biopsies [9]. Temozolomide has shown beneficial effects in patients with relapsed SCLC, especially in a subgroup associated with the presence of the MGMT promoter methylation [10]. The drug has shown promising activity in patients with glio(-blast-)oma and relapsed SCLC, with a response rate of 22% in all comers, of 19% in third-line and of 38% in patients with brain metastases [10]. It is hypothesized that the presence of MGMT promoter methylation in NETs and NECs may act as a predictive marker for response to treatment with temozolomide [11].

The *ALK* fusion gene is mostly formed by a rearrangement occurring on the short arm of chromosome 2 and involves the genes encoding for ALK (2p23.2) and echinoderm microtubule-associated protein-like 4 (EML4) (2p21) [12]. Several other translocation partners have been described. Rearrangement of ALK occur in a variety of tumors, including NSCLC, anaplastic large cell lymphomas, inflammatory myofibroblastic tumors and neuroblastomas [13–15]. Little is known about ALK rearrangement in NETs and NECs [16]. As ALK plays an important role in the development of the brain and in specific neurons in the nervous system, we hypothesize that ALK expression or translocation is present in tumors of the neuroendocrine crest [17]. ALK rearrangement can act as biomarker for the treatment with ALK inhibitors in this selected patient group. Repurposing of drugs used for other indications and/or tumor types is an acceptable and innovative strategy in the advancement of treatment of these devastating carcinomas. We performed a retrospective analysis on tissue samples of patients with NETs and NECs to establish the frequency of MGMT promoter methylation and the frequency of ALK expression and rearrangement.

RESULTS

Patients and tumor classification

After approval by the local Scientific board of the local Biobank and of the Ethical Committee and having obtained the consent of the patients, we collected from the local biobank the archival samples of 74 treatment-naïve patients who were diagnosed as NETs and NECs between January 2014 and December 2016, data of the hospital electronic system were retrospectively collected. Their characteristics such as age stage, diagnosis, performance score and treatment were extracted from their medical records. In case of surgically removed NETs, the primary tumor was tested for ALK and MGMT promoter methylation. In case of metastasized SCLC or LCNEC most samples were from metastases, either lymph node samples or metastases in other organs (except brain and bone metastases). Pathological diagnoses of these 74 patients were verified from archival tissue and made according to the World Health Organization classification based on morphology [4]. Confirmation of the pathologic diagnosis was made by a dedicated pathologist (PP) and was performed on IHC with synaptophysin, chromogranin A, and Ki-67. Tumors were classified as NETs, typical carcinoid (grade 1) and atypical carcinoid (grade 2) tumors, to the NECs grade 3 and 4, SCLC and LCNEC variants with a high metastatic potential and a poor prognosis.

Most patients had metastatic SCLC. Patients characteristics and test results are described in Table 1. There was adequate tissue available for ALK testing in 70 patients and in 67 patients for MGMT promoter methylation testing. Patients were treated according to the

guideline. Patients were not treated with an ALK-inhibitor in case of positive ALK-IHC, nor with temozolomide if presence of MGMT promoter methylation as both treatments were not available as reimbursed medication.

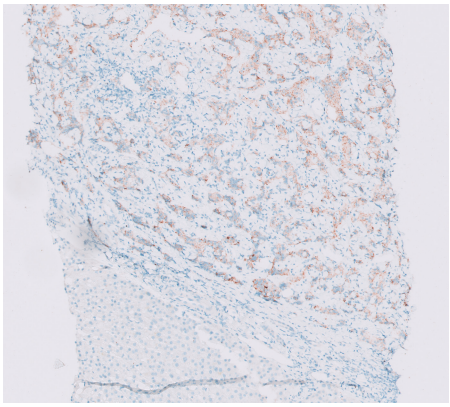
Table 1. Patient characteristics and results of ALK and MGMT testing

	N = 74	ALK IHC positive	ALK IHC negative	MGMT positive	MGMT negative
Evaluable cases		10	60	14	53
Age range (years)	39 - 88	46 – 88	39 - 88	45 - 88	39 - 88
Gender					
Male	40 (54%)	4	36	7	26
Female	34 (46%)	6	28	7	27
Disease stage					
I	17	3	13	4	11
II	7	0	6	2	4
III	20	3	16	4	15
IV	30	4	25	4	23
Tumor histology					
Typical carcinoid	10	2 (22%)	7 (78%)	3 (33%)	6 (67%)
Atypical carcinoid	9	2 (25%)	6 (75%)	2 (22%)	7 (78%)
SCLC	41	6 (15%)	33 (85%)	8 (22%)	29 (78%)
LCNEC	14	0 (0%)	13 (100%)	1 (8%)	11 (92%)

ALK: anaplastic lymphoma kinase; IHC: immunohistochemistry; MGMT: O6-Methylguanine-DNA methyltransferase; NET: neuroendocrine tumor; NEC: neuroendocrine carcinoma; SCLC: small cell lung carcinoma; LCNEC: large cell neuroendocrine carcinoma.

Analysis of ALK IHC, FISH and ALK mutations

Ten of 70 (14%) specimens were ALK IHC positive (Table 1). The ten ALK IHC positive specimens consisted of two typical carcinoids, two atypical carcinoids, and six SCLC. None of the 13 LCNECs were ALK IHC positive. ALK IHC positive specimen were tested for ALK FISH (Figure 1). None of them showed rearrangements. In 5 tissues of high ALK expression the presence of *ALK* mutations was tested, but no *ALK* mutations were present.



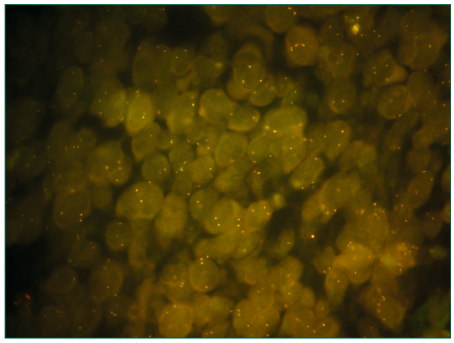


Figure 1: (A) ALK IHC staining (10X): moderate to strong staining in 70% of the tumor cells. (B) ALK FISH: ALK IHC positive SCLC sample without ALK rearrangement (only fused signals present)

Analysis of the MGMT promoter methylation testing

In 67 of 74 patients, tissue was sufficient for evaluation. In 21% (14/67) of patients tested positive for MGMT promoter methylation (Table 1). MGMT promoter methylation was present in 33% (3/9) patients with typical carcinoid, in 22% (2/9) patients with atypical carcinoid, in 22% (8/37) patients with SCLC and in 8% (1/12) patient with LCNEC.

DISCUSSION

This is one of the largest series with NET and NEC where the role of MGMT promoter methylation and ALK is studied.

In our series we found ALK expression in 14% of a cohort of 70 patients with NETs and NECs. No ALK- rearrangement was present. These findings are in accordance with the largest series of patients with SCLC published [18–20] (Table 2). To evaluate whether ALK expression in NET/NEC is generally associated with *ALK* mutations we selected five patient samples with a high ALK expression for *ALK* mutation analysis. We made the assumption that if ALK-mutations were absent in the high-expressors, patients with a low or negative ALK expression harbored no *ALK* mutations. None of the specimens with a high ALK expression *ALK* mutations were detected. Kondoh et al., examined specimens of 142 patients with SCLC, 41 patients with LCNEC and 11 patients with carcinoids [18]. In the SCLC cohort, ALK expression was detected in 16 of 142 (11.3%) and 4 of 12 specimens were found to carry copy gain numbers. In the LCNEC and carcinoid cohort no rearrangements, no amplifications, no point mutations and no ALK expression was found. No significant association was found between ALK expression and overall survival. The authors conclude that ALK expression in SCLC was due to intrinsic expression of a normal ALK transcript.

In another series aberrant ALK expression in 227 pulmonary NECs was observed in 2 (2.9%) of 69 SCLC and 1 (0.9%) of 106 LCNEC [19]. In 52 carcinoid tumors no ALK expression was observed. In three ALK positive NECs no ALK rearrangement nor amplification was found, also no *ALK* mutation was detected. In a smaller series of 32 LCNEC tumors, no ALK expression was seen. Nor were ALK fusions or *ALK* mutations detected [20]. This data is in agreement with our results demonstrating that ALK expression is not associated with the presence of an ALK rearrangement or *ALK* mutation.

A number of case-reports on ALK rearrangements in atypical carcinoid, SCLC and LCNEC have been reported (Table 2). Not all specimen were tested for ALK expression, and no *ALK* mutations were revealed. Two cases of SCLC were reported containing an ALK rearrangement in a series of 30 patients with SCLC [16, 21]. Both cases had a combined SCLC with an ALK expression and an adenocarcinoma. In the first case presented the adenocarcinoma component harbored an *EGFR* mutation, deletion in exon 19 [16]. It was stated by the authors that adenocarcinomas with an *EGFR* mutation can transform into SCLC in the process of acquiring resistance to EGFR tyrosine kinase inhibitors. As this patient didn't receive any medication before diagnosis, the mechanism rather reflects coincidence than transformation as acquired resistance. In the second case report a patient with SCLC harboring a variant 2 of the *EML4-ALK* fusion gene [21]. This SCLC was repeatedly confirmed by histological biopsy, however stained negative for TTF1 and positive for ALK. The patient showed a partial response on chemotherapy. After progression, a biopsy confirmed SCLC with ALK expression. The patient didn't receive treatment with an ALK inhibitor. In another case of combined SCLC and adenocarcinoma, an *ALK* gene alteration was found in both components [22]. In a case report of combined SCLC and atypical carcinoid no testing for ALK was conducted [23]. These patients received standard of care, none of these patients were treated with ALK inhibitors. We hypothesize that these findings in combined SCLC and adenocarcinoma or a combined SCLC and atypical carcinoid suggests that the origin of the lung tumor may be monoclonal. We did not include combined tumors in our series.

Table 2. Studies on ALK in NETs and NECs

		Samples (n)	ALK expression	ALK rearrangement	ALK mutation, amplification, copy gain number (CGN)	Response on ALK inhibitor
Kondoh [18]	SCLC	142	16 (11.3%) 0	Not present	CGN: 4/12 (33,3%) Not present	No response of crizotinib in in-vitro cell lines
	LCNEC	41	0	Not present	Not present	
	Typical carcinoid	11		Not present		
Nakamura [19]	SCLC	69	2 (2.9%)	Not present	NA	NA
	LCNEC	106	1 (0.9%)	Not present		
	Carcinoid	52	0	Not present		
Karlsson [20]	LCNEC	32	NA	Not present	NA	NA
Toyokawa [16]	SCLC and adenocarcinoma	1	Present	Variant 1 EML4 – ALK fusion	NA	NA
Toyokawa [21]	SCLC and adenocarcinoma	1	Present	Variant 2 EML4 – ALK fusion	NA	NA
Bai [22]	SCLC and adenocarcinoma	1	Present	*KLC1* – ALK fusion	NA	NA
Pronk [23]	SCLC and atypical carcinoid	1	NA	NA	NA	NA
Wang [24]	Atypical carcinoid	1 (liquid biopsy)	NA	*SMC5* – ALK fusion	Not present	Alectinib, partial response
Nakajima [25]	Atypical carcinoid	1	Present	Present	NA	Crizotinib, partial response
Fukuizumi [26]	Atypical carcinoid	1	Present	Variant 3a/b EML4 – ALK fusion	Not present	Crizotinib, no response
Omachi [27]	LCNEC	1	Present	Variant 2 EML4 – ALK fusion	NA	Crizotinib, progressive disease
Miyoshi [28]	SCLC	1	Present	Not present	NA	NA
Hayashi [29]	LCNEC	1	NA	Present	NA	Alectinib, response

ALK: anaplastic lymphoma kinase; NET: neuroendocrine tumor; NEC: neuroendocrine carcinoma; SCLC: small cell lung carcinoma; LCNEC: large cell neuroendocrine carcinoma; NA: not analyzed.

Case reports of NETs or NECs that were treated with ALK-inhibitors showed different responses. One case of atypical carcinoid with an ALK-rearrangement showed partial response on alectinib after progression on temozolomide and capecitabine [24]. Another patient with an atypical carcinoid with ALK expression and ALK rearrangement progressed after chemotherapy and was successfully treated with crizotinib [25]. An atypical carcinoid with variant 3a/b ALK rearrangement did not respond to crizotinib [26]. Crizotinib as first generation ALK inhibitor maybe less powerful. A case report of a patient with LCNEC with ALK rearrangement responding to alectinib after progression on chemotherapy [27]. In a case of advanced LCNEC expressing ALK on IHC and an ALK rearrangement with FISH, the patient treated with crizotinib in first line [28]. The first evaluation six weeks later showed progressive disease. The conclusion is that ALK rearrangement may not be of practical importance in LCNEC and that neuroendocrine tumors with ALK rearrangement may be less responsive to ALK inhibitors. This stresses the importance to assess *ALK* fusion genes with FISH or NGS (RNA) in case of ALK expression [29].

In our series standard testing for ALK was done by FISH testing, as it was – at that time – the standard test considered 'gold standard'. In later times we tested ALK IHC as the abnormal ALK protein product of fusion genes may be associated with elevations in ALK protein, detectable by IHC. A positive ALK expression is considered sufficient indication for treatment with an ALK inhibitor in NSCLC. Currently, superior second and third generation ALK inhibitors are available with a better systemic and intracranial efficacy than crizotinib, which was used in some of the patients in the case reports. As sporadic cases of TKI-addicted ALK altered lung cancers are in the NET/NEC population, selected patients fit enough for advanced line therapy, should be tested for the presence of ALK protein.

MGMT promoter methylation in NETs and NECs

Epigenetic alterations in cancer are a potential source of predictive therapeutic biomarkers for personalized cancer treatment. Whereas MGMT promoter methylation may have predictive value, MGMT expression by IHC does not [30].

A feasibility study was conducted in relapsed SCLC to evaluate the MGMT promoter methylation in tissue, cytology and sputum [9]. Of 56 patients with SCLC, 30 tissue biopsies, 17 fine-needle aspirates, 8 bronchial washings and 1 sputum were available. Methylation analysis was obtained in 54 samples (and failed in two bronchial washings). MGMT promoter methylation was detected in 35.2% without any significant difference between histological and cytological samples (37.9% vs. 32%) (Table 3). The assay used for MGMT analysis is an in house developed validated assay for glioblastoma samples.

The assay is highly suitable for glioblastoma samples as annual EQA schemes for central nervous system tumors demonstrate good results. Although the assay works well for small tissue fragments and cytology, the assay has not been validated on SCLC/NET/NEC samples. The degree of MGMT methylation is a continuous value and the ideal cut-off value for hypermethylation of SCLC/NET/NEC might be different than in central nervous system tumor. Another limitation is that when a partial loss of both chromosomes 10 occurs, the MGMT assay can produce a false negative result because this loss is not taken in account.

No prospective data about the incidence of MGMT promoter methylation is available in lung NETs. In other retrospective series, MGMT is methylated in 0–27% of lung NETs [31, 32]. This outcome is comparable to our series.

To our knowledge, one report is available describing MGMT promoter methylation in LCNEC samples. This study revealed the presence of MGMT promoter methylation in 2 of 6 patients [31]. Our retrospective series contains a larger patient group, but in only 1 patient out of 12 (9%) MGMT promoter methylation was detected.

Table 3. Studies on MGMT promoter methylation in NETs and NECs

		Samples histology (n)	Samples cytology (n)	MGMT promoter methylation (%)	Response on temozolomide
Miglio [9]	SCLC	30	24	35.2	NA
Pietanza [10]	SCLC	27		48	RR 38%
Zauderer [37]	SCLC	8		87.5	1 PR (14%)
Walter [11]	Carcinoid	5		80	NA
Pietanza [39]	SCLC	32		31	Not significantly
Lei [31]	Carcinoid and LCNEC	12		16.6	NA
Lu [38]	SCLC	33		51.5	NA

MGMT: O6-Methylguanine-DNA methyltransferase; NET: neuroendocrine tumor; NEC: neuroendocrine carcinoma; SCLC: small cell lung carcinoma; LCNEC: large cell neuroendocrine carcinoma; NA: not analyzed; RR: response rate; PR: partial response.

MGMT promoter methylation is significantly associated with tumor response to temozolomide in glioblastoma multiforme and NETs [10]. Recent guidelines recommend temozolomide treatment in advanced unresectable progressive pulmonary atypical carcinoid tumors [33]. The optimal dosing regimen and schedule with temozolomide is still under debate [34]. Treatment with temozolomide is an option in relapsed advanced

SCLC [35], however, the only approved second-line treatment in relapsed SCLC is topotecan [36].

The efficacy of temozolomide was reported in several studies (Table 3). The sample size is too small to estimate a pooled response rate on temozolomide in the MGMT promoter methylation positive patients. Pietanza et al., studied 64 patients with progressive SCLC after one or two prior chemotherapy regimens who received temozolomide at 75 mg/m2 daily for 21 days of a 28-day cycle [10]. The primary endpoint was response rate. The tumor response of 22% was observed in an unselected group, in third line the tumor response was 19%. In those with brain metastases the tumor response was 38%. In 48% (n = 27) of patients, a MGMT promoter methylation was detected. The response rate to temozolomide was 38% in the MGMT promoter methylated group versus 7% in the group without MGMT promoter methylation, suggesting that a tumor response due to temozolomide may be associated with the presence of MGMT promoter methylation. Twenty-five patients were enrolled in a single center trial of a 5-day dosing regimen of temozolomide 200 mg/m2 in a 28-day cycle [37]. The rationale for this shortened dosing schedule was to avoid prolonged myelosuppression. The primary endpoint, tolerability, was met with common toxicity criteria grade 3 and 4 toxicity in 5 out of 25 patients. Temozolomide was well-tolerated. Responses were seen in 12 patients (48%, 95% CI: 3–31%). No responses in the brain were seen with this regimen. Eight tissues were tested for the MGMT promoter methylation and of these, 7 had evidence of promoter methylation of whom 1 had a partial response. The small sample size does not allow to draw solid conclusions about the predictive value of MGMT promoter methylation.

In another study, 17 out of 33 Chinese SCLC patients (51.5%) had MGMT promoter methylation [38]. A comparative study between temozolomide and veliparib versus temozolomide with placebo in patients with relapsed SCLC did not show improved progression free survival [39]. Analysis of MGMT promoter methylation as a biomarker was limited, as sufficient DNA was available in only 32 of 104 tumor samples. The MGMT promoter was methylated in 31% (7 of 32) of the samples tested and was not associated with tumor response or with improved progression free survival or overall survival.

Our series revealed MGMT promoter methylation in 22% of patients with SCLC. As SCLC is a recalcitrant illness, there is an unmet need for treatment options in relapsed or refractory disease. As guidelines recommend treatment with temozolomide, stratification by MGMT promoter methylation can select a patient group that benefits from temozolomide. We propose a prospective study in which a biomarker selected patient group with MGMT promoter methylation is treated with temozolomide.

MATERIALS AND METHODS

Neuroendocrine protein expression

Confirmation with immunohistochemistry (IHC) was performed with neuroendocrine markers such as synaptophysin, chromogranin A. Ki-67 expression was used as proliferation marker. IHC was performed with synaptophysin (clone DAK-SYNAP, RTU, Agilent), chromogranin A (Clone LK2H10, 1/500, Menarini), and Ki-67 (Clone MIB-1, RTU, Agilent) on an Autostainer Link 48 instrument (Agilent) using the Envision Flex detection kit (Agilent).

ALK Immunohistochemistry

Subsequently, these samples were analyzed for ALK expression. FFPE sections (5-µm thickness) were stained using ALK 5A4 (Leica) with EnVison Flex+, mouse high pH detection reagents on an Autostainer Lin 48 instrument (Dako, Glostrup, Denmark). The sections were subsequently incubated in high pH buffer (20 min, 97°C; PT-Link, Dako), peroxidase blocking buffer (5min), primary antibody (1:50, 30 min), mouse-enhanced polymer-based linker (30 min), mouse secondary antibody (20 min), diaminobenzidene (5 min) and haematoxylin (5 min) as previously described [40]. ALK expression was assessed independently by one pathologist (PP) and one scientist (KZ). IHC ALK positive samples were evaluated with Fluorescence in-situ hybridization (FISH). High ALK expressors were analyzed with next generation sequencing to detect ALK mutations.

ALK Fluorescence in-situ hybridization

FISH was performed on 5-µm formalin-fixed, paraffin-embedded (FFPE) tissue sections using the Vysis ALK dual-color, break-apart rearrangement probe in combination with the Vysis pre-and post-treatment kit IV (Abbott Molecular, Des Plaines, IL, USA) according to the manufacturer's instructions. Results were analyzed using a fluorescent BX41 microscope (Olympus, Center Valley, PA, USA) and evaluated according to the Vysis LSI ALK Probe manufacturer's enumeration guidelines.

Fluorescence in-situ hybridization (FISH) with Vysis/Abbott LSI ALK probe was performed in IHC positive cases. ALK FISH was considered positive if at least 15% op tumor cells showed rearrangement (50 nuclei were evaluated).

ALK mutation analysis

ALK mutation analysis was performed using an in house developed and validated Next Generation Sequencing panel detecting single nucleotide variations and small indels in genomic DNA of amongst other exon 22, 23 and 25 of the ALK gene with a sensitivity of 3%. From each sample 10 unstained slides of 5 µM thickness were prepared. Upon

macro dissection of the tumor region gDNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen) on a Qiacube instrument. Upon HaloPlex enrichment (Agilent) of the target DNA sequencing analysis was executed on a MiSeq platform using the MiSeq Reagent kit V2 (300 cycles) (Illumina). Analysis of the data was performed using the JSI SeqNExt v4.1.2 software.

Methylation specific PCR of the promoter region of MGMT

MGMT promoter methylation was analyzed with PCR. Upon macro dissection, DNA isolation of FFPE section was performed using the QIAamp DNA blood mini kit (Qiagen). Bisulfite-mediated conversion of the extracted gDNA was performed using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. TaqMan qPCR assay was performed on this converted gDNA to amplify MGMT and ACTB with the following primers and probes: MGMT-FWD 5'-GCGTTTCGACGTTCGTAGGT-3', MGMTREV 5'-GCACTCTTCCGAAAACGAAACG-3', MGMT-PROBE 5'-FAM-CGCAAACGATACGCACCGCGA, ACTB-FWR 5'-TGGTGATGGAGGAGGT TTAGTAAGT, ACTB-REV 5'-AACCAATAAAACCT ACTCCTCCCTTAA-3', ACT-PROBE 5'-VIC-ACCAC CACCCAACACACAATAACAAACACATA-3'. These primer and probe sequences were obtained from Parella et al., and Esteller et al., [41, 42]. Amplification was performed using the LightCycler 480 probes master mix (ROCHE) on a Cobas 4800 platform with an hybridization temperature of 60°C. In each run, a non-template control, a WT control and a positive control (2.5% U87D cell line in background of tonsil FFPE tissue) was included. The MGMT assay has been validated on glioblastoma samples with a detection limit of 1%. The assay is suited for both tissue and cytology samples and required a ratio of neoplastic cells of minimally 10%. The assay is ISO15189 accredited and annual participation to EQA scheme (GENQA CNS schemes) consistently demonstrated good results.

Samples were deemed informative if the Cp value of the ACTB gene was <31 and the samples were scored positive when for MGMT a Cp value of <36 was obtained. These Cp values were established and verified by respectively comparing the results with the assay described by Esteller et al., [42] and with other Belgian and Dutch diagnostic, accredited laboratories performing MGMT analysis in routine practice.

CONCLUSIONS

A subset of NETs and NECs stains positive for ALK-IHC. As protein expression of ALK is especially found in neuronal tissue like thalamus, hypothalamus, midbrain and dorsal root ganglia, the question is whether altered ALK present in neuroendocrine tumors of the lung, can act as a target for treatment with ALK inhibitors. Although 14% of patients expressed ALK, rearrangement was absent. Since mutations in ALK tyrosine

kinase domain have also be described to cause ALK expression, also *ALK* mutation analysis was performed. However, no *ALK* mutations were found. We suggest that ALK expression reflects the origin of the tumor, the neuroendocrine crest. In absence of an ALK rearrangement there's no indication for treatment with an ALK inhibitor.

A sizable fraction of patients NEC and NET present with a MGMT promoter hypermethylation, which is considered a driver alteration for targeted treatment with temozolomide. Prospective data are needed, preferably in a randomized design. We hence recommend testing refractory or relapsing patients with NEC and NET for the presence of this alteration on archival tissue, in order to ascertain their eligibility for such a treatment.

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Chapter 6

Circulating tumor DNA as a biomarker for monitoring early treatment responses of patients with advanced lung adenocarcinoma receiving immune checkpoint inhibitors

Paul van der Leest*
Birgitta Hiddinga*
Anneke Miedema
Maria L. Aguirre Azpurua
Naomi Rifaela
Arja ter Elst
Wim Timens
Harry J.M. Groen
Léon C. van Kempen
T. Jeroen N. Hiltermann
Ed Schuuring
*contributed equally

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ABSTRACT

Immunotherapy for metastasized non-small-cell lung cancer (NSCLC) can show long-lasting clinical responses. Selection of patients based on programmed death-ligand 1 (PD-L1) expression shows limited predictive value for durable clinical benefit (DCB). We investigated whether early treatment effects as measured by a change in circulating tumor DNA (ctDNA) level is a proxy of early tumor response to immunotherapy according to response evaluation criteria in solid tumors v1.1 criteria, progression free survival (PFS), DCB, and overall survival (OS). To this aim, blood tubes were collected from advanced-stage lung adenocarcinoma patients (n = 100) receiving immune checkpoint inhibitors (ICI) at baseline (t0) and prior to first treatment evaluation (4–6 weeks; t1). Nontargetable (driver) mutations detected in the pretreatment tumor biopsy were used to quantify tumor-specific ctDNA levels using droplet digital PCR. We found that changes in ctDNA levels were strongly associated with tumor response. A > 30% decrease in ctDNA at t1 correlated with a longer PFS and OS. In total, 80% of patients with a DCB of ≥ 26 weeks displayed a > 30% decrease in ctDNA levels. For patients with a PD-L1 tumor proportion score of ≥ 1%, decreasing ctDNA levels were associated with a higher frequency a DCB (80%) and a prolonged median PFS (85 weeks) and OS (101 weeks) compared with patients with no decrease in ctDNA (34%; 11 and 39 weeks, respectively). This study shows that monitoring of ctDNA dynamics is an easy-to-use and promising tool for assessing PFS, DCB, and OS for ICI-treated NSCLC patients.

INTRODUCTION

Treatment with immune checkpoint inhibitors (ICI) for advanced non-small-cell lung cancer (NSCLC) patients without targetable genetic alterations demonstrated long-lasting therapy response and overall survival (OS) in selected patients [1–5]. Programmed death-ligand 1 (PD-L1) protein expression in the pretreatment tumor tissue determines eligibility for immunotherapy targeting PD-1 or PD-L1 inhibitors with or without chemotherapy. First-line treatment with pembrolizumab is currently standard of care for patients with advanced NSCLC. However, even in patients with tumors having a high PD-L1 expression (≥ 50% of tumor cells), a durable clinical benefit (DCB) of treatment is achieved in less than half of the cases [3,6,7]. Nivolumab monotherapy as treatment beyond first line resulted in 4-year OS of 14% (95% confidence interval [CI]: 11–17%) for all patients (n = 664), 19% (95% CI: 15–24%) for those with at least 1% PD-L1 expression, and 11% (95% CI: 7–16%) for those with less than 1% PD-L1 expression [4]. Although eligibility criteria for immunotherapy are defined, there is an urgent demand for improved predictive and prognostic biomarkers that define which patients benefit from treatment. The ability to identify non responders at an early stage of ICI treatment could avoid severe toxicities associated unnecessary continuation of ICI treatment and reduce the financial burden on the healthcare system.

Solely relying on tumor PD-L1 expression has proven clear limitations to accurately predict tumor response assessment by response evaluation criteria in solid tumors (RECIST) v1.1 criteria [8]. Furthermore, early on-treatment radiologic assessment of tumor response cannot always predict durability of response because patients with initial pseudoprogression or stable disease (SD) may have durable responses comparable to patients who do have a radiological tumor response [4]. Therefore, a biomarker that better predicts or can monitor treatment effects for individual patients, alone or in combination with PD-L1, is increasingly demanded [9]. Recent studies showed that monitoring the circulating tumor-derived DNA (ctDNA) fraction in the circulating cell-free DNA (ccfDNA) in plasma samples, as a surrogate for biological tumor response, correlates with individual early tumor responses and clinical outcome to treatment in several cancer types [10,11], including NSCLC patients treated with ICI using expensive and complex nextgeneration sequencing (NGS) methodologies on serial plasma ccfDNA [12–16].

Droplet digital PCR (ddPCR) analysis of plasma ctDNA is routinely used for clinical applications to detect targetable mutations in epidermal growth factor receptor (EGFR) [17–19], KIT [20], and BRAF [21,22], with an analytical sensitivity of 0.1–0.01% and specificity > 99% [19,20,22,23]. Here, we focused on a sensitive ddPCR test to monitor

changes in ctDNA in plasma from advanced lung adenocarcinoma patients receiving single-agent ICI. For this study, the target ctDNA was selected from the Pathology archives that reported on clinically relevant mutations determined by NGS analysis of the primary tumor in routine clinical practice. Patients with tumors harboring a nontargetable somatic mutation such as pathogenic mutations in kirsten rat sarcoma viral oncogene homolog (*K-ras*), and who were therefore treated with single-agent ICI, were prospectively included. In addition to patients with *K-ras* mutations, patients with non-*K-ras* mutated tumors (e.g., *BRAF* and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations) were included to rule out *K-ras* mutation-specific observations. To date, only three other studies with relatively small cohorts of advanced NSCLC patients treated with ICI selected tumor-informed nontargetable somatic mutations for monitoring ctDNA levels using a single-gene assay [24–26]. Here, we investigated changes in ctDNA levels as a proxy of early tumor response to ICI for progression free survival (PFS), DCB, and OS in cohort of 100 patients with advanced lung adenocarcinoma using this approach.

MATERIAL AND METHODS

Patient selection

Patients were recruited between October 2015 and November 2019. In total, 100 patients with advanced adenocarcinoma receiving ICI treatment were eligible for this study. Mutation analysis via NGS of the pretreatment formalin-fixed paraffin-embedded (FFPE) tissue biopsies was performed in the routine diagnostic setting. These results were available for this study. Follow-up data for all patients were obtained up to the database lock (October 9, 2020). Eligibility criteria were ≥ 18 years of age, Eastern Cooperative Oncology Group performance-status score (ECOG PS) ≤ 1, advanced-stage adenocarcinoma and measurable disease assessed by means of computed tomography (CT) according to RECIST v1.1 [27]. This study is a larger cohort based on CA209-759 study (NTR 6158) and was approved by the Medical Ethical Committee (METc, 2010/109) of the University Medical Center Groningen (UMCG). The study methodologies were conformed to the standards set by the Declaration of Helsinki. All patients provided written informed consent.

Radiological evaluation

Positron emission tomography/CT imaging was assessed at baseline in all patients. Tumor evaluation with CT was performed every 6 weeks in the first year of ICI treatment, thereafter every 12 weeks until disease progression. RECIST v1.1 criteria were used to assess tumor response. CtDNA dynamics were used to predict radiological response and DCB. Progressive disease (PD) is defined as an increase in tumor volume of > 20%

or appearance of new lesions. Partial response (PR) is defined as a decrease in tumor volume of > 30%; complete response (CR) as response showing that all lesions (both target and nontarget) are less than 10mm in the long axis (except lymph nodes which have to be smaller than 10mm in short axis). SD is attributed if neither the criteria for PD, PR or CR are met.

Plasma collection and ccfDNA extraction

Blood samples were available in either vacutainer EDTA tubes (vacutainer #367525, Becton Dickinson, Franklin Lakes, NJ, USA; until December 2017) or cell-free DNA blood Streck collection tubes (BCTs; Streck, Omaha, NE, USA), since January 2018. Processing of cell-free plasma and ccfDNA extraction was according to standard operating procedure as reported previously [28,29]. In short, EDTA blood samples were processed within 4 h and Streck samples within 24 h. Subsequent processing consisted of a slow (for EDTA: 820 g, 10 min, 4 °C; for Streck: 1600 g, 10 min, 20 °C) and subsequent fast (16 000 g, 10 min, 4 °C) centrifugation step. Plasma was stored as 1 mL aliquots at 80 °C until ccfDNA extraction. CcfDNA was extracted from ~ 2 mL plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and as reported previously [28]. CcfDNA was eluted in 52 µL of AVE buffer and its concentration was measured by QubitTM dsDNA HS assay kit on a QubitTM 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

To determine the most appropriate timepoint after start ICI therapy to measure changes in ctDNA levels, a subset of 27 patients was first selected from whom plasma was stored of several timepoints between baseline and disease progression, as well as four patients who displayed rapid disease progression (within 6 weeks; Table S1). For this subset, 164 plasma samples were collected with on average 6 (2–12) samples per patient. After the appropriate timepoint of follow up was established, all 100 patients were analyzed at baseline (t0) and at 4- to 6-week follow-up (t1).

Tumor specimen handling and tissue NGS

As routine workup of suspected lung cancer, tumor tissue was obtained by a bronchoscopy, transthoracic biopsy or an endoscopic ultrasound procedure (endobronchial ultrasound/endoscopic ultrasound). Tissue samples were processed and diagnosed following routine pathology procedures. Following Dutch guidelines, FFPE-pretreatment tissue samples of all adenocarcinomas from patients with metastasized NSCLC were subjected to sequence analysis by targeted NGS for mutations in relevant predictive markers including *EGFR*, *BRAF*, *K-ras*, *PIK3CA*, erb-b2 receptor tyrosine kinase 2 and *MET* [30] in the NEN-EN-ISO15189-accredited laboratory for molecular pathology at the UMCG as reported previously [20,31]. Molecular results are reported in the Dutch

nationwide pathology registry (PALGA). For this study, lung adenocarcinoma patients were selected with a somatic mutation for which no targetable drugs were available and therefore were treated with ICI (see Table 1 for overview of mutations). Out of 22 patients with non-*K-ras* mutations, 11 patients with a targetable mutation (e.g., *BRAF* V600E, *EGFR* L858R, or *EGFR* T790M) were included following progression on tyrosine kinase inhibitors (TKIs) or as a last resort treatment. PDL1 expression was detected with the Ventana PD-L1 (SP263) Assay (RTU, conformité Européene-in vitro diagnostic) on a Ventana Benchmark Ultra immunostainer on pretreatment tissue biopsies. Staining was scored by an experienced pulmonary pathologist (WT) according to international classification criteria and reported as tumor proportion score (TPS) for 87 patients [32].

Quantitative ctDNA analysis

For each patient, a tumor-specific ddPCR assay using nontargetable (driver) mutations present in the pretreatment biopsy was selected in order to detect and quantify the tumor-specific mutations in ccfDNA (Table S2). DdPCR analysis was performed as reported previously [20,23,28]. In short, ccfDNA (median 5.4 ng, 1.3–61 ng) was emulsified into 10.000– 20.000 droplets by the QX200TM droplet generator (Bio-Rad Laboratories, Pleasanton, CA, USA) and amplified with ddPCRTM supermix (Bio-Rad) and the primers and probes (Table S2) into a final volume of 20 µL. Mutant (FAM-labelled) or wild-type (HEX-labelled) fluorescent quantitative signals were detected by the QX200TM platform (Bio-Rad). DdPCR results were analyzed with QuantaSoftTM analytical software (Bio-Rad). Droplet counts were used to calculate the number of mutant copies per mL of plasma. The variant allelic frequency was determined by QuantaSoftTM Analysis Pro. Samples were regarded as positive if ≥ 3 mutant droplets were detected and negative if < 3 mutant droplets with at least 330 total positive (wild-type and mutant) droplets were detected (ensuring an analytical sensitivity < 1%). Because previous assessments of the precision of the ddPCR tests that are used in this study revealed a 30% technical variance [23], we set the minimum threshold at 30% and we only consider changes in mutant ctDNA levels greater than 30% as a true increase or decrease. In addition, we evaluated more stringent thresholds of 40% and 50% that were previously reported to be informative [12,33,34]. To confirm the changes in ctDNA levels detected with ddPCR, a fully automated real-time PCR IdyllaTM ctKRAS Mutation Assay (Biocartis, Mechelen, Belgium) was performed as reported previously [35,36]. All analyses included mutation-positive, wild-type, and no template controls. All standard precautions were taken to avoid contamination of amplification products using separate laboratories for pre- and post-PCR handling. Clinical and laboratory test outcomes were independently added into the database.

Statistical analysis

Descriptive statistics were used for patient and tumor characteristics. PFS and OS were defined as the period between the date of start of ICI to the date of PD or date of death, respectively. Data were censored at the date of last follow-up in absence of an event. Kaplan– Meier survival data were stratified for mutant ctDNA data and compared with the log-rank test. To compare ctDNA dynamics with PD-L1 TPS, Kaplan–Meier curves were stratified according to the PD-L1 TPS. Radiological reports and liquid biopsy test results were assessed independently. Correlation between the *K-ras* G12/13 screening ddPCR assay and Idylla™ ctKRAS Mutation Assay results was determined using Pearson's correlation coefficient and agreement was performed using Cohen's κ. Differences in the rate of DCB were assessed with a Mann–Whitney U test. GRAPHPAD PRISM 8.4.2 (GraphPad Software, San Diego, CA, USA) or SPSS version 25 software (IBM SPSS Statistics, Armonk, NY, USA) were used for all statistical analysis, wherein a P-value < 0.05 was considered significant.

RESULTS

Patient characteristics

Next-generation sequencing analysis of the pretreatment FFPE tissue biopsies identified 78 tumor samples with mutations in *K-ras* (78%) and 22 with a non-*K-ras* mutation (22%). All clinical and pathological characteristics are summarized in Table 1. Most patients (n = 69) were treated with nivolumab 3 mg/kg body weight intravenously every 2 weeks or pembrolizumab 200 mg (n = 28 patients) every 3 weeks intravenously (Table 1). In addition, two patients were treated with atezolizumab 1200 mg every 2 weeks and one patient with durvalumab 20 mg/kg every 2 weeks. The median number of weeks from start ICI until tumor response was 6 weeks (2–55 weeks). Follow-up CT imaging was not performed in eight patients (8%) as clinical PD already occurred prior to the first radiological evaluation. Sixty-six patients (66%) had an early tumor response, defined by a tumor response according to RECIST v1.1 within 6 weeks after start ICI treatment. A late tumor response, defined by tumor response according to RECIST v1.1 after 12 weeks, was observed in 18 (18%). A DCB is defined by a clinical response with at least SD lasting ≥ 6 months as reported previously [8], which was achieved in 39 patients (39%).

Table 1: Clinical and pathological characteristics

Patients	**100**
Median age	66 (29-85)
Male	53
Female	47
ECOG PS	
0	42
1	49
2	7
3	2
Smoking status	
Current	39
Former	58
Never	3
Immunotherapy	
Atezolizumab	2
Durvalumab	1
Nivolumab	69
Pembrolizumab	28
Previous lines of (chemo)therapies	
0	25
1	57
2	12
3	6
***K-ras* mutations**	78
c.35G>C p.(G12A)	4
c.34G>T p.(G12C)	37
c.35G>A p.(G12D)	9
c.34G>C p.(G12R)	1
c.35G>T p.(G12V)	18
c.37G>T p.(G13C)	1
c.38G>A p.(G13D)	3
c.183A>C p.(Q61H)	3
c.181C>A p.(Q61K)	1
c.182A>T p.(Q61L)	1
Non-*K-ras* mutations	22
BRAF c.1397G>C p.(G466A)	1
BRAF c.1397G>T p.(G466V)	2
BRAF c.1406G>C p.(G469A)	3
BRAF c.1406G>T p.(G469V)	1
BRAF c.1799_1801del p.(V600_K601delinsE)	1
BRAF c.1799T>A p.(V600E)	5
EGFR c.2310_2311insGGC p.(D770_N771insG)	1
EGFR c.2155G>A p.(G719S)	1
EGFR c.2316_2321dup p.(H773_V774dup)	1
EGFR c.2573T>G p.(L858R)	1
PIK3CA c.1624G>A p.(E542K)	3
PIK3CA c.1633G>A p.(E545K)	2

Patients	100
PD-L1 TPS	
<1%	34
1-49%	17
≥50%	35
N/A	14

ECOG PS: Eastern Cooperative Oncology Group performance status score; PD-L1 TPS: programmed death-ligand 1 tumor proportion score; N/A: not available.

Optimal timepoint to measure changes in ctDNA levels associated with durable tumor response

Twenty-seven patients with a *K-ras* or *BRAF* (nonV600E) mutation in the primary tumor from whom plasma was available at several timepoints during ICI treatment, predominantly at 1, 2, 4, and 6 weeks after initiation, were selected (Table S1) to determine the optimal timepoint to measure changes in ctDNA levels associated with therapy response effects. CcfDNA was analyzed to quantify mutant ctDNA copies. Tumor response patterns could be divided into five typical patterns for CR, PR, SD, PD, and ctDNA negative patients (see examples in Fig. S1A–E). The ctDNA patterns of all responding patients (n = 11) revealed an initial spike in ctDNA levels prior to a decrease in ctDNA levels (Fig. S2A). One exceptional case is discussed separately (Fig. S3). In samples at 4–6 weeks, most of the responders (70–89%) showed a > 30% decrease, while in most of the nonresponders (55– 75%) ctDNA levels at 4–6 weeks increased (Fig. S2B). Spider plot analysis supported the predictive value of ctDNA analysis 4–6 weeks after start therapy (t1). Patients with increased, stable or nondetectable (considered as negative) levels of ctDNA demonstrated early disease progression, of whom 14/16 (88%) have deceased. The majority of patients with decreasing ctDNA demonstrated a response, of whom 10/11 (91%) were alive after at least 80 weeks (Fig. 1).

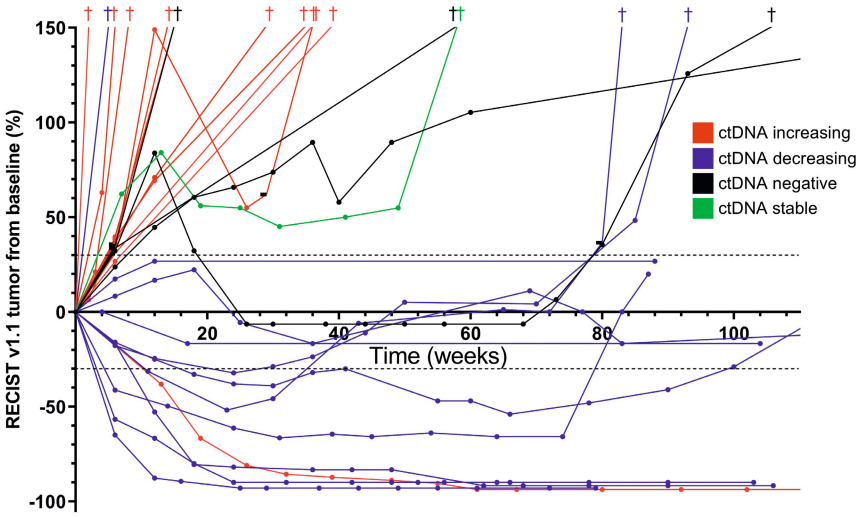

Figure 1. Spider plot analysis of radiological response according to the RECIST v1.1 criteria and changes in mutant ctDNA levels

CtDNA levels were determined by the difference in mutant copies per mL of plasma at baseline (t0) and 4–6 weeks after start of ICI treatment (t1). Dashed lines indicate a 20% increase and 30% decrease in tumor volume compared with baseline. The cross symbol indicates the patient's death at that point in time. One exceptional case is described in Fig. S3. CtDNA increasing, 30% more mutant copies at t1 compared with t0; ctDNA decreasing, 30% less mutant copies at t1 compared with t0; ctDNA-negative, driver mutation in tissue not detected in plasma; ctDNA stable, observed change in mutant copies at t1 compared with t0 was ≤ 30%.

Validation of KRAS ddPCR analysis with Idylla ctKRAS

To confirm the levels of KRAS-mutated ctDNA detected in cell-free plasma using ddPCR analysis, 89 samples with sufficient plasma were also analyzed with the Idylla™ ctKRAS Mutation Assay as an independent plasma-based test. Based on the number of mutant copies per mL plasma, ddPCR and Idylla revealed similar results (r^2 = 0.94, black line; r^2 = 0.64 omitting six cases with very high levels, blue line; Fig. S4). When comparing changes in KRAS mutant ctDNA levels between t0 and t1, 13 of the 15 patients showed a similar association with clinical response represented by an almost perfect agreement when comparing ddPCR with Idylla (κ = 0.84). These data confirmed that quantitative ctDNA analysis using ddPCR reliably predicted changes in mutant ctDNA levels.

Changes in ctDNA levels as an early marker of durable clinical benefit

To validate the potential value of monitoring ctDNA levels, ddPCR analysis was performed on ccfDNA from 100 lung adenocarcinoma patients treated with mono-immunotherapy. When ctDNA was detected at t0, a significant difference in the number of mutant

copies per mL of plasma was observed between patients with no clinical response and patients who had a DCB (Fig. S5A). Patients with high mutant ctDNA levels at t0 showed a poorer PFS ($P < 0.001$) and OS ($P < 0.0001$) compared with low mutant copy levels (Fig. S5B,C). No ctDNA was detected at t0 in 31 patients (31%). CtDNA-negative patients were represented both in 21 of the 63 non responders (33%) and 10 of 37 of durable responders (27%) (Fig. S5A, red dots).

Patients with a decrease in ctDNA levels at t1 had the best median PFS and OS (Fig. 2A,B). Patients with both stable ctDNA (change at t1 compared with t0 ≤ 30%) or increased (> 30%) ctDNA levels showed similar poor responses. Therefore, patients with a ctDNA increase or ctDNA stable levels were grouped as no ctDNA decrease in subsequent analyses. Although 70% of patients without detectable ctDNA (16/23) showed early disease progression (within 6 months), they did perform better than patients with increasing or stable levels of ctDNA, but worse than those with a decrease in ctDNA was observed at t1 (Fig. 2B). Therefore, patients without detectable ctDNA were regarded as a separate group.

Analysis excluding ctDNA-negative patients revealed that patients with decreasing mutant ctDNA levels had a significantly improved PFS (hazard ratio [HR]: 0.41 [0.19–0.52]; $P < 0.0001$) compared with patients who did not (no decrease in mutant ctDNA), resulting in a longer median PFS (43 vs 6 weeks; Fig. 2C) and OS (125 vs 29 weeks; HR: 0.32 [0.16–0.46]; $P < 0.0001$; Fig. 2D). Using a higher threshold of 50% for ctDNA response (Fig. S6) revealed comparable results as observed for 30% with only a slightly improved HRs for PFS and OS.

To exclude that the observed association between ctDNA levels and treatment response was due to the specific activity of *K-ras* mutations, the PFS and OS comparing presence (n = 78) or absence (n = 22) of *K-ras* mutations in the pretreatment tumor tissue were evaluated. This analysis revealed no significant difference in PFS and OS (Fig. S7).

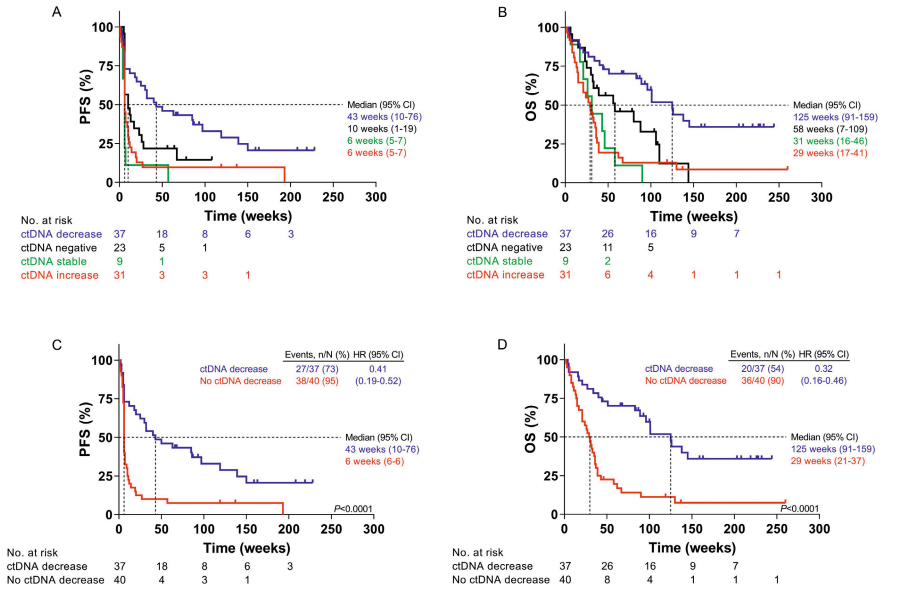

Figure 2. Tumor response related to changes in mutant ctDNA levels

Kaplan–Meier plot displaying the (A) PFS and (B) OS of patients with decreasing (blue), negative (black), stable (green), or increasing (red) ctDNA levels. (C) PFS and (D) OS of patients with decreasing ctDNA levels (blue), or no decrease in ctDNA (red). Log-rank test, P-values of < 0.05 are considered significant. CtDNA decreasing, 30% less mutant copies at t1 compared with t0; ctDNA-negative, driver mutation in tissue not detected in plasma; ctDNA stable, observed change in mutant copies at t1 compared with t0 was ≤ 30%; ctDNA increasing, 30% more mutant copies at t1 compared with t0; no decrease in ctDNA, encompasses patients with ctDNA increase and ctDNA stable.

PD-L1 expression in pretreatment tissue biopsies and ctDNA dynamics

PD-L1 expression data were available for 87 patients. Thirty-five patients (40%) were PD-L1 negative (TPS < 1%) and 52 (60%) had a PD-L1 TPS ≥ 1% (of whom 35 with TPS ≥ 50%; Table 1). In this cohort, patients with a PD-L1 TPS of ≥ 1% had a longer PFS (25 vs 6 weeks; HR: 0.46 [0.22–0.61]; P < 0.001) and OS (83 vs 32 weeks; HR: 0.57 [0.32–0.92]; P < 0.05) than PD-L1 negative patients (Fig. S8). In patients with a PD-L1 TPS of ≥ 1%, decreased ctDNA levels further improved both PFS (85 vs 11 weeks; HR: 0.42 [0.22–0.78]; P < 0.01) and OS (101 vs 39 weeks; HR: 0.37 [0.19–0.72]; P < 0.01; Fig. 3A, B; Fig. S9A,B). Interestingly, in a subset of PD-L1- negative patients (TPS of < 1%), decreased ctDNA levels were also associated with prolonged PFS and OS (Fig. 3C,D; Fig. S9C,D). The effect of a ctDNA decrease on PFS was stronger for patients with PD-L1 expressing tumors compared with patients with PDL1-negative tumors (HR: 0.40 [0.14–0.80], P < 0.05; data not shown).

DISCUSSION

When a tumor-derived molecular aberration is detected in plasma, this can potentially be used to monitor early tumor response to ICI. In the current study, we demonstrate the value of measuring ctDNA levels using ddPCR at baseline (t0) and follow-up (4–6 weeks, t1) as a minimally invasive monitoring tool for response to ICI monotherapy. The group of patients who displayed a decrease in mutant copies had a longer PFS, OS, and DCB compared with those without decrease in ctDNA levels. Furthermore, patients who displayed a reduction in mutant tumor DNA in circulation and had a PD-L1 expressing tumor demonstrated an even better PFS, OS, and DCB. The data indicate that the combination of PD-L1 expression and reduction in ctDNA is a stronger monitoring tool for response to ICI than PD-L1 expression or change in ctDNA alone.

Detection of tumor-derived DNA in liquid biopsy has enabled assessment of mutation profiles in plasma of cancer patients at different stages of disease in a minimally invasive manner [37]. Recent studies advocate NGS of pretreatment plasma samples as the most appropriate approach to identify mutants for disease monitoring of virtually all patients. Subsequently, a selection of these mutations can be monitored in plasma over time. In current clinical practice however, high cost of plasma-derived ccfDNA NGS for all patients is cost-prohibitive. In contrast, it is currently common practice to perform molecular profiling on a tumor tissue biopsy with broader NGS mutation panels. Mutation profiling of tumor biopsies not only resulted in the identification of clinical-relevant druggable targets, but also in tumor-specific variants that may be detected in circulation. In the current study, the tumor-informed ddPCR analysis of ctDNA has demonstrated promise as a cost-effective monitoring tool.

We studied dynamics of mutant ctDNA levels prior to radiological evaluation in plasma using mutations that were detected in the pretreatment tissue biopsies as part of routine molecular diagnostics. In the first 2 weeks of treatment, a spike in ctDNA levels was observed in 61% of all patients with measurable ctDNA at baseline (14/23), and in 70% of patients who eventually demonstrated treatment response (Fig. S2). This transient spike in ctDNA was reported previously for KRAS and EGFR in NSCLC, probably reflecting tumor DNA release by death of tumor cells upon initiation of systemic treatment [11,12,38]. The strong increase in ctDNA within 2 weeks after start of therapy that was observed in 15 patients was not predictive for DCB (data not shown). Our analysis demonstrated that at least a 30% decrease in ctDNA levels at 4–6 weeks after initiation of treatment (t1) correlated with a longer PFS and OS in response to ICI treatment, as well as an increased rate of DCB (Table S3). A decrease in mutant ctDNA levels was associated with a superior median PFS (43 weeks, HR: 0.41 [0.19–0.52]) and OS (125 weeks, HR: 0.32 [0.16– 0.46])

compared with that of combined patient group with increasing or stable ctDNA levels (PFS 6 weeks; OS 29 weeks). These results are comparable to three other studies with small cohorts of advanced NSCLC patient (respectively 14 [24], 34 [25], and 15 cases [26]) with non-targetable mutations detected in tumor biopsy treated with ICI. Despite that KRAS-mutated tumors were associated with high PD-L1 expression and consequently with increased tumor responses toward PD- (L)1 inhibition [2,39–41], no discrepancies between tumor harboring *K-ras* or other mutations were observed in our cohort.

In the current study, the median PFS of patients with a PD-L1 TPS ≥ 1% is just 25 weeks. Further dividing PD-L1 TPS in 1–49% and ≥ 50%, which is generally applied in current literature, did not reveal significant differences regarding PFS (P = 0.22) and OS (P = 0.15; data not shown). Combining independent biomarkers has previously shown to augment the predictive potential for DCB, as previously shown for plasma NGS with CD8+ cell levels [14]. When combining PD-L1 immunohistochemistry in pretreatment tumor biopsies with changes in ctDNA levels, these changes did not correlate with PD-L1 TPS, indicating that both markers are independent biomarkers (Fig. S10). In fact, combining changes in ctDNA with PD-L1 TPS ≥ 1% showed an eightfold longer PFS and more than twofold longer OS in patients with a decrease in ctDNA levels compared with patients who did not show a > 30% decrease (Fig. 3). A subset of patients with a PD-L1 TPS of < 1% with decreasing ctDNA levels seems to benefit from monotherapy as well (Table S4). Responders to immunotherapy in our study were observed both with high and low PD-L1 tumors. The value of ctDNA decrease for monitoring treatment effect was independent of PD-L1 expression. Reck et al. [3] also reported an improved response upon decrease in ctDNA at t1 in a patient cohort with PD-L1 expression for first-line ICI treatment using a cutoff of TPS ≥ 50%. In line with this observation, evaluation of patients with PD-L1 TPS ≥ 50% and a decrease in ctDNA revealed even lower HRs (0.32 for PFS and 0.29 for OS; data not shown). However, in the current study 75% of patients was not treatment naïve. Patients who received previous lines of treatment generally show poorer response and survival times to ICI [4]. Despite the low number of patients in this study, this underscores the strong monitoring potential of change in ctDNA in combination with or without PD-L1 expression and warrants further prospective evaluation. The sensitivity of this combination monitoring tool might further be augmented by addition of other potentially predictive biomarkers such as the immunoscore, immune infiltration, cytokine signatures (e.g., interferon gamma, transforming growth factor beta), and somatic copy number alterations [42–44].

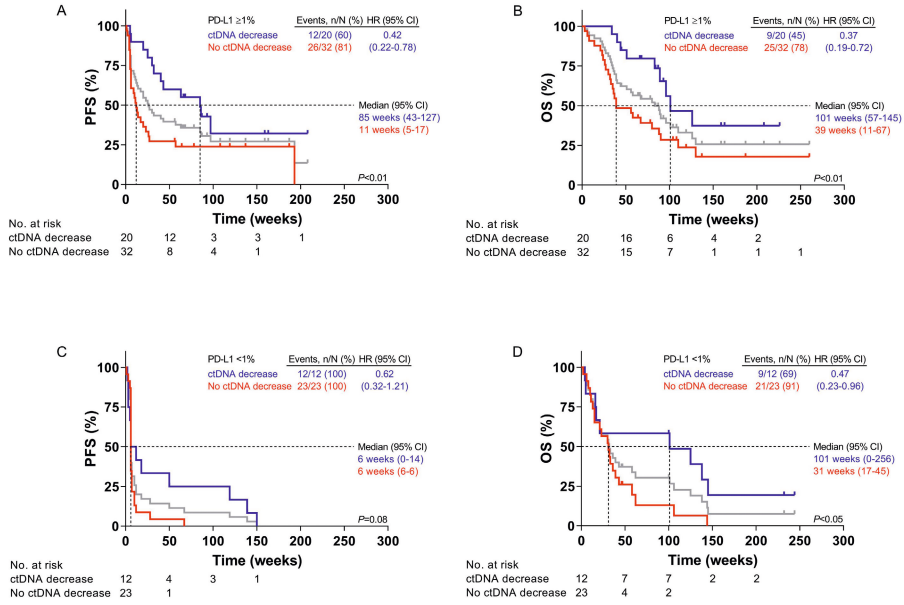

Figure 3. Tumor response related to change in mutant ctDNA levels and PD-L1 TPS status

Kaplan-Meier plot displaying the (A, C) progression-free survival (PFS) and (B, D) overall survival (OS) of patients with a PD-L1 TPS of ≥1% (A-B) and <1% (C-D) with decreasing (blue), or increasing or stable (red) ctDNA levels. The grey lines represent the entire PD-L1 cohort in the respective subgroups (not used in comparison of the different subgroups). Supplementary Figure 9 shows the analysis of patients with decreasing, stable, increasing and non-detectable ctDNA levels separately. Log-rank test, P-values of <0.05 are considered significant. CtDNA decreasing, 30% less mutant copies at t1 compared to t0; No decrease in ctDNA, encompasses patients with ctDNA increase, ctDNA negative and ctDNA stable; HR, hazard ratio; CI, confidence interval.

In patients with known driver mutations, these mutations are not retrieved in approximately 30% of matched cell-free plasma in various malignancies [45]. In line with these observations, in 31% of the included patients with metastasized disease the mutation detected in the pretreatment tumor biopsy was not detected in the corresponding ccfDNA sample at t0. No ctDNA was detected in 23% of the patients at both timepoints. Although the majority of patients without detectable ctDNA did not display a tumor response to treatment, their tumors seemed to have a more indolent course than those who did have specific ctDNA. This group of patients did have early PD in general, but OS was markedly better than for the ctDNA group showing stable levels or an increase at t1. The cause of absence of ctDNA in these plasma remains uncertain and proposed mechanisms include nonshedding tumors, increased clearance, shorter half-life, lack of sufficient analytical sensitivity, and stage of disease [37, 45].

To monitor tumor response in ccfDNA using mutation-specific ddPCR analysis, sequencing of pretreatment tumor tissue is required to select a tumor-specific target. In 50% of advanced-stage NSCLC targetable (~ 20%) or nontargetable *K-ras* (~ 30%) driver mutations are detected with current commonly-used diagnostic NGS approaches [31, 45]. However, mutations detected in the tumor may not always be present in plasma. Broadening routine clinical tissue NGS panels, for example, with the frequently mutated TP53 and STK11 genes, will increase the number of patients who can be effectively monitored for tumor response using plasma ccfDNA with single gene approaches such as ddPCR. In this study, five patients with tumors containing multiple mutations at least one of these mutations could not be detected in the plasma. Selection of a mutation for monitoring purposes in plasma might lead to inconsistent results (Table S5). As such, several studies in lung cancer advocate the use of NGS analysis with a broad panel of markers on baseline plasma samples instead of a single selected marker. Targeting multiple mutations simultaneously also elevates the sensitivity of detecting ctDNA [12, 46]. Indeed, the number of ctDNA negative patients when using NGS approaches is substantially lower (4–8%) than was observed with our single variant assay [14, 47]. Studies that used an NGS approach to monitor ctDNA in response to ICI therapy demonstrate a correlation between ctDNA dynamics and response similar to our findings [12, 13, 48]. Recently, three studies comprising larger cohorts of various malignancies including NSCLC treated with ICI reported on the association between serial ctDNA NGS testing and PFS, OS, clinical response, and clinical benefit [14–16]. However, in current clinical practice, NGS approaches on ccfDNA are not yet cost-effective for monitoring the course of treatment longitudinally. Single-target ddPCR analysis therefore provides a cost-effective alternative when the ctDNA target is detectable in the circulation. Longitudinal monitoring of a single tumor-derived variant beyond the currently proposed interval might assist in early detection of disease progression and its clinical applicability, probably in combination with multiple available biomarkers, should be investigated in future (prospective) studies. Besides, as ccfDNA is shed into circulation from various tissues, DNA fragments from hematopoietic and germline origin are prone to affect analytical results with NGS, as well as inconsistent preanalytical handling and sample processing [23,49– 51]. Although the majority of clonal hematopoietic variants occur in non-targetable genes, these variants are also identified in targetable genes such as KRAS, BRAF, and PIK3CA as well [16]. Deep sequencing of plasma may therefore identify more mutations, but these might not all be derived from the tumor. To this extent, parallel sequencing of a patient-matched bloodborne reference material, for example, white blood cells, is of importance [50], further increasing the costs for routine clinical practice. Therefore, monitoring ctDNA with a ddPCR assay is as sensitive as NGS to monitor therapy response but in a cost-effective manner. However, ddPCR is only informative when tumor-derived DNA is present in circulation.

CONCLUSION

Altogether, decreasing mutant copies estimated with ddPCR were associated with longer PFS and OS compared with patients displaying increased or stable ctDNA levels. CtDNA dynamics in combination with PD-L1 status is a promising cost-effective approach to monitor DCB, PFS, and OS in patients treated with ICI. Measuring a single tumor-derived molecular aberration, when retrieved in the circulation, improves the early recognition of DCB and can assist in treatment decision making.

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SUPPORTING INFORMATION

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Supplementary Table 1. Overview of subgroup of 27 patients to determine the most appropriate timepoint after start ICI to measure clinically relevant changes in ctDNA

Patient ID	Tumor mutation	RECIST v1.1 response	Timepoints available plasma samples (weeks after start of treatment)
B-001	*KRAS* c.38G>A p.(G13D)	PD	0, 3*
B-002	*BRAF* c.1406G>C p.(G469A)	PR	0, 2, 4, 6, 12, 24, 36, 48, 60, 72, 84, 96
B-003	*KRAS* c.34G>T p.(G12C)	PR	0, 1, 2, 13, 24, 34, 48, 61, 72, 84, 96, 108
B-004	*KRAS* c.35G>C p.(G12A)	SD	0, 1, 2, 4, 6, 12
B-005	*KRAS* c.35G>A p.(G12D)	PR	0, 1, 5, 9, 13, 36
B-006	*KRAS* c.34G>T p.(G12C)	PR	0, 1, 2, 4, 6, 24, 36, 48, 60
B-007	*KRAS* c.183A>C p.(Q61H)	PD	0, 1, 2, 4
B-008	*KRAS* c.34G>T p.(G12C)	PD	0, 2, 4*
B-009	*KRAS* c.35G>A p.(G12D)	PD	0, 1, 2, 4, 6
B-010	*KRAS* c.35G>C p.(G12A)	PD	0, 2, 4, 6
B-011	*BRAF* c.1406G>C p.(G469A)	SD	0, 1, 2, 4, 6, 12
B-012	*KRAS* c.34G>T p.(G12C)	PD	0, 1, 4, 6
B-013	*KRAS* c.35G>A p.(G12D)	PR	0, 1, 2, 4, 6, 12
B-014	*KRAS* c.35G>A p.(G12D)	PR	0, 1, 2, 4, 6, 12
B-015	*KRAS* c.35G>T p.(G12V)	SD	0, 1, 2, 4, 6, 12, 24, 36, 60
B-016	*KRAS* c.34G>T p.(G12C)	SD	0, 1, 2, 4, 6, 12, 24, 36, 48, 74
B-017	*KRAS* c.34G>T p.(G12C)	PD	0, 1, 6, 70
B-018	*KRAS* c.34G>T p.(G12C)	SD	0, 1, 2, 4, 6, 12
B-019	*KRAS* c.35G>T p.(G12V)	PD	0, 1, 2, 4, 6
B-020	*KRAS* c.34G>T p.(G12C)	PD	0, 1*
B-021	*KRAS* c.34G>T p.(G12C)	CR	0, 1, 2, 4, 6, 12, 23, 37, 46, 58
B-022	*KRAS* c.34G>T p.(G12C)	PD	0, 1, 2, 4, 13
B-023	*KRAS* c.35G>T p.(G12V)	PD	0, 5*
B-024	*KRAS* c.34G>T p.(G12C)	PR	0, 1, 2, 4
B-025	*KRAS* c.34G>T p.(G12C)	CR	0, 1, 2, 4, 6, 8, 12, 24, 36
B-026	*KRAS* c.35G>T p.(G12V)	PR	0, 1, 2, 7, 24
B-027	*KRAS* c.35G>T p.(G12V)	PD	0, 1, 2, 4, 6

*Patients with rapid disease progression (within 6 weeks). RECIST, Response Evaluation Criteria in Solid Tumors; PD: progressive disease (non-response); SD: stable disease (non-response); PR: partial response; CR: complete response.

Supplementary Table 2. Assays used for ddPCR analysis

Bio-Rad assay name	Bio-Rad assay ID	Targeted nucleotide sequence
BRAF p.G466A	dHsaMDS389209582	NM_004333: *BRAF* c.1397G>A
BRAF p.G466V	dHsaMDS2510966	NM_004333: *BRAF* c.1397G>T
BRAF p.G469A	dHsaMDV2516932	NM_004333: *BRAF* c.1406G>C
BRAF p.G469V	dHsaMDS747800353	NM_004333: *BRAF* c.1406G>T
BRAF p.V600E	dHsaMDV2010027	NM_004333: *BRAF* c.1799T>A
BRAF p.V600_K601>E	dHsaMDS890722866	NM_004333: *BRAF* c.1799_1801delTGA
EGFR p.D770_N771insG	dHsaMDS625148063	NM_005228: *EGFR* D770_N771insG
EGFR p.G719S	dHsaMDV2010041	NM_005228: *EGFR* c.2155G>A
EGFR p.V774_C775insHV c.2315_2316insCCACGT*	dHsaMDS712821910	NM_005228: *EGFR* c.2315_2316insCCACGT
EGFR p.L858R c.2573T>G	dHsaMDV2010021	NM_005228: *EGFR* c.2573T>G
EGFR p.T790M	dHsaMDV2010019	NM_005228: *EGFR* c.2369C>T
ddPCR *KRAS* G12/G13 Screening Kit	1863506	†
ddPCR *KRAS* Q61 Screening Kit	12001626	‡
PIK3CA p.E542K	dHsaMDV2010073	NM_006218: *PIK3CA* c.1624G>A
PIK3CA p.E545K	dHsaMDV2010075	NM_006218: *PIK3CA* c.1633G>A

Assay IDs are displayed as provided by Bio-Rad Laboratories Inc. *Annotation of the *EGFR* p.V774_C775insHV assay according human genome variation society (HGVS) is *EGFR* p.(H773_V774dup). †The ddPCR *KRAS* G12/G13 Screening Kit was used to screen cases with a *KRAS* c.35G>C p.(G12A), c.34G>T p.(G12C), c.35G>A p.(G12D), c.34G>C p.(G12R), c.34G>A p.(G12S), c.35G>T p.(G12V) or c.38G>A p.(G13D) mutation. ‡The ddPCR *KRAS* Q61 Screening Kit was used to screen cases with a *KRAS* c.181C>A p.(Q61K), c.182A>T p.(Q61L), c.182A>G p.(Q61R), c.183A>T p.(Q61H) or c.183A>C p.(Q61H) mutation.

Supplementary Table 3. CtDNA dynamics and clinical response

	PD	SD	PR	CR	NCR (<6 months)	DCB (≥6 months)
ctDNA decrease	10 (27%)	5 (14%)	16 (43%)	6 (16%)	14 (38%)	23 (62%)*
No decrease in ctDNA	30 (75%)	4 (10%)	6 (15%)	0 (0%)	35 (88%)	5 (12%)
ctDNA negative	12 (52%)	8 (35%)	2 (9%)	1 (4%)	16 (70%)	7 (30%)

62% of the patients with decreased mutant copies display a DCB, as opposed to 12% of the patients with increasing or stable ctDNA levels (*P=0.0001, Mann-Whitney U test comparing PFS of patients with ctDNA decrease with no decrease in ctDNA. Although many patients without detectable mutant ctDNA levels both at t_0 and t_1 (n=23) demonstrated early disease progression, 30% achieved a DCB. PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response; NCR: no clinical response; DCB: durable clinical benefit.

Supplementary Table 4. CtDNA dynamics and PD-L1 TPS score

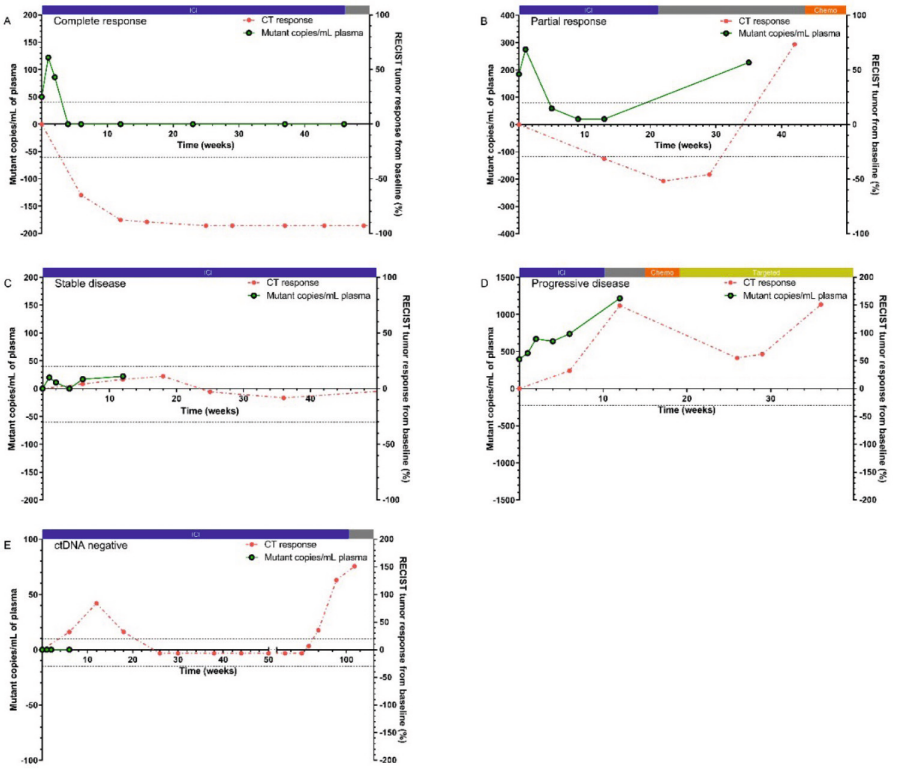
		PD	SD	PR	CR	NCR (<6 mo)	DCB (≥6 mo)
TPS ≥1%	ctDNA decrease	2 (10%)	1 (5%)	12 (60%)	5 (25%)	4 (20%)	16 (80%)*
	No decrease in ctDNA	13 (59%)	3 (14%)	6 (27%)	0 (0%)	17 (77%)	5 (23%)
	ctDNA negative	3 (30%)	4 (40%)	2 (20%)	1 (10%)	4 (40%)	6 (60%)
TPS <1%	ctDNA decrease	6 (46%)	4 (31%)	2 (23%)	0 (0%)	8 (62%)	5 (38%)†
	No decrease in ctDNA	15 (100%)	0 (0%)	0 (0%)	0 (0%)	15 (100%)	0 (0%)
	ctDNA negative	5 (63%)	3 (37%)	0 (0%)	0 (0%)	5 (63%)	3 (38%)

Of the patients with decreasing mutant copies, 80% achieved a DCB, while 77% of patients displaying stable or ctDNA increase did not respond to treatment (*P<0.001, Mann-Whitney U test comparing PFS of patients with ctDNA decrease with no decrease in ctDNA). No significant difference in response rate was observed for patients with a PD-L1 TPS <1% (†P=0.31, Mann-Whitney U test comparing PFS of patients with ctDNA decrease with no decrease in ctDNA). TPS: PD-L1 Tumor Proportion Score; PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response; NCR: no clinical response; DCB: durable clinical benefit; mo: months.

Supplementary Table 5. Patients with multiple targetable mutations

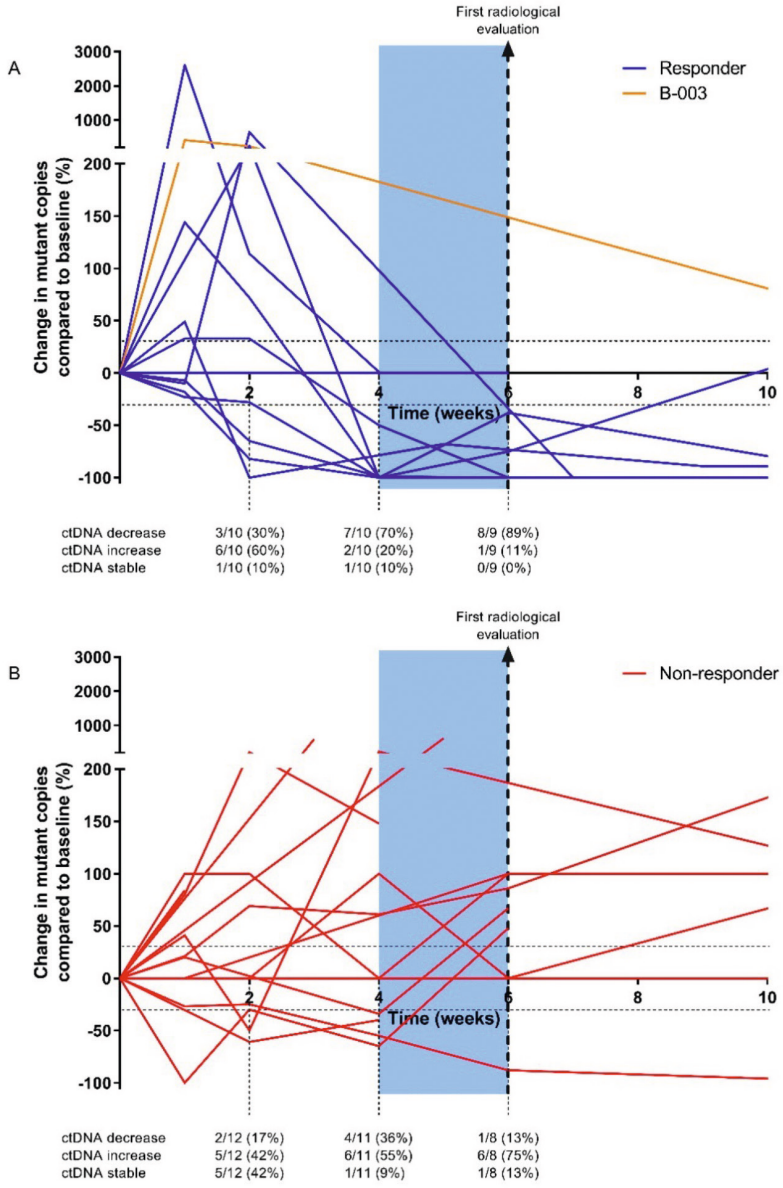
	Tissue		Plasma mutant copies/mL			Radiology
Patient	**Mutation**	**VAF (%)**	**t_0**	**t_1**	**ctDNA change**	**RECIST v1.1**
B-026	*KRAS* c.35G>T p.(G12V)	39	49.3	0.0	Decrease	PR
	PIK3CA c.1624G>A p.(E542K)	12	0.0	0.0	Negative	
B-032	*EGFR* c.2573T>G p.(L858R)	58	407	178	Decrease	PR
	EGFR c.2369C>T p.(T790M)	3	0.0	0.0	Negative	
B-065	*KRAS* c.34G>T p.(G12C)	11	727	632	Stable	PD
	BRAF c.1397G>T p.(G466V)	10	0.0	0.0	Negative	
B-072	*PIK3CA* c.1633G>A p.(E545K)	8	0.0	0.0	Negative	SD
	PIK3CA c.1624G>A p.(E542K)	8	0.0	0.0	Negative	
B-098	*BRAF* c.1397G>T p.(G466V)	51	55.4	0.0	Decrease	PR
	BRAF c.1799T>A p.(V600E)	5	0.0	0.0	Negative	

VAF: variant allelic frequency; mutant copies/mL: mutant copies per mL of plasma; RECIST: Response Evaluation Criteria in Solid Tumors; PD: progressive disease; SD: stable disease; PR: partial response.



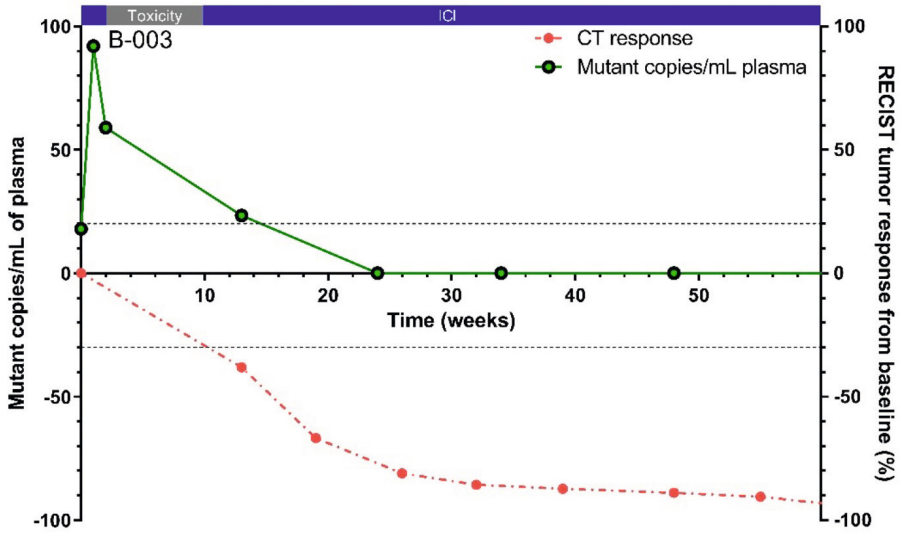
Supplementary Figure 1. Patterns of response combining mutant ctDNA levels and tumor volume using CT scanning

Display of the radiological response compared to baseline (t_0, red) and changes in ctDNA levels (green) during treatment. Red dots and green circles represent timepoints that respectively CT-scanning was performed or plasma sample was drawn. Dashed lines indicate a 20% increase and 30% decrease in tumor volume compared to baseline. The bar on top shows which treatment the patients received over time, either immune checkpoint inhibitors (ICI, blue), chemotherapy (chemo, orange), targeted therapy (targeted, yellow) or no treatment (gray). Representative patterns are shown for patients displaying a (A) complete response (CR), (B) partial response (PR), (C) stable disease (SD), and (D) progressive disease (PD). (E) Most ctDNA negative patients showed progressive disease in an early stage, however did survive for a long time.



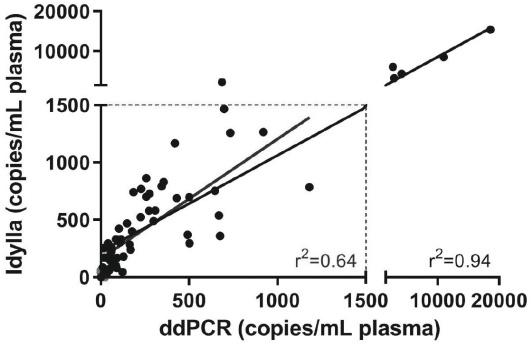
Supplementary Figure 2. Determination of most appropriate timepoints to optimally detect changes in ctDNA levels related to treatment response

Representation of the percentage change in ctDNA levels over time compared to baseline for responders (A, blue) and non-responders (B, red) according to the RECIST v1.1 criteria (see Supplementary Table 1). Dashed lines indicate a 20% increase and 30% decrease in tumor volume compared to baseline. Appropriate timepoints to detect changes in ctDNA prior to the first radiological evaluation are between 4-6 weeks, as most responders have decreased ctDNA levels (A) and most non-responders have increase ctDNA levels (B). Patient B-003 (A, orange) defined as a responder is discordant but lacks plasma samples between 2-12 weeks after treatment for proper interpretation (see Supplementary Figure 3). Data of four patients were not included as mutant ctDNA were not detected (negative cases), one responder and three non-responders.



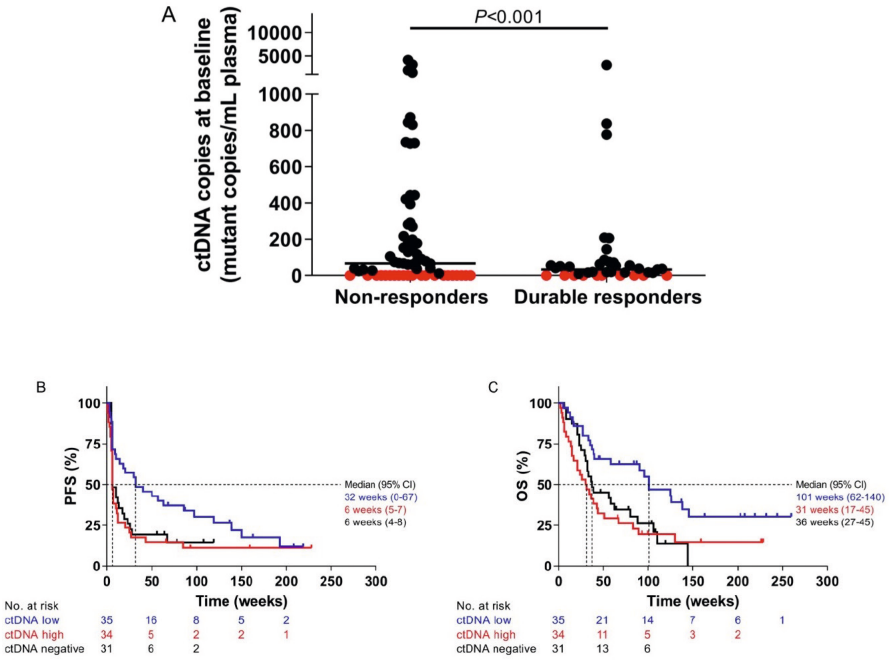
Supplementary Figure 3. Evaluation of case B-003

Change in mutant copies compared to baseline (t_0) is presented over time. Dashed lines indicate a 20% increase and 30% decrease in tumor volume compared to baseline. The bar on top indicates the patient received immune checkpoint inhibitors (ICI, blue) the entire follow-up time, except from 2-10 weeks after initiation when treatment was stopped due to toxicity (gray). In this patient, an initial spike in ctDNA was observed in the first two weeks of treatment. No blood sample was collected between 2-12 weeks after start of treatment. At 24 weeks, the patient has complete clearance of ctDNA and showed a consistent response to treatment.



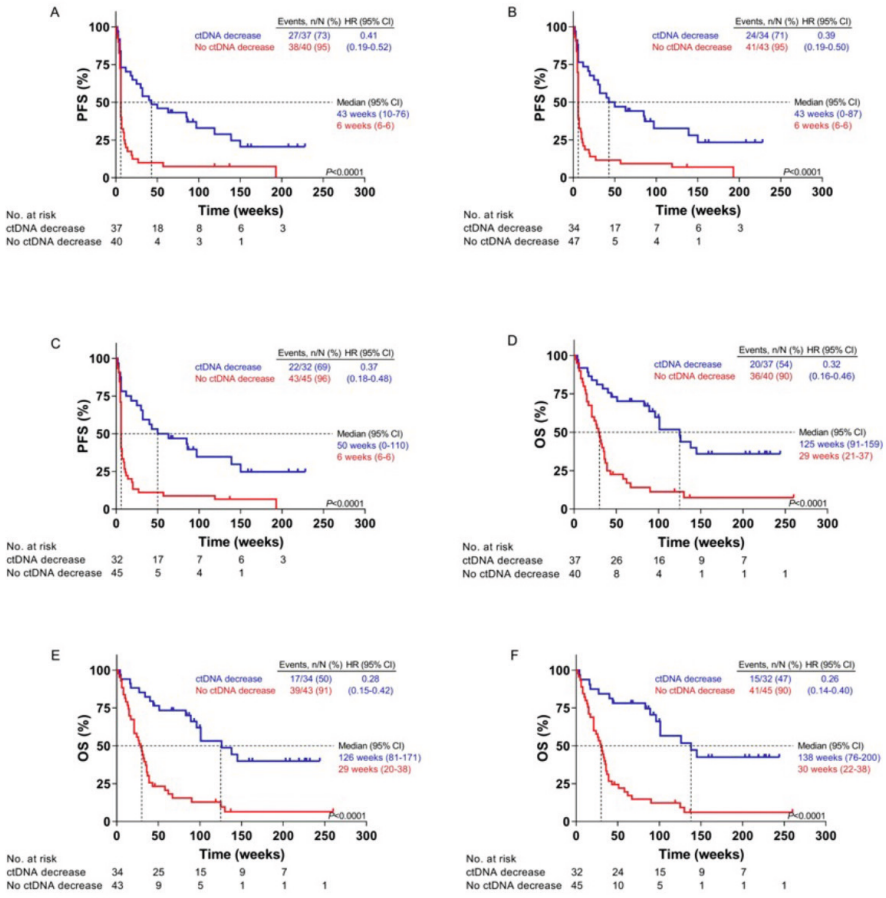
Supplementary Figure 4. Correlation of the mutant copies per mL of plasma as determined with ddPCR and Idylla™ ctKRAS Mutation Assay

Red-colored results were not included in the correlation due to failure of either mutation detection assay. Correlation was determined for all samples (black line, r^2=0.94) and excluding outliers (blue line, r^2=0.64).



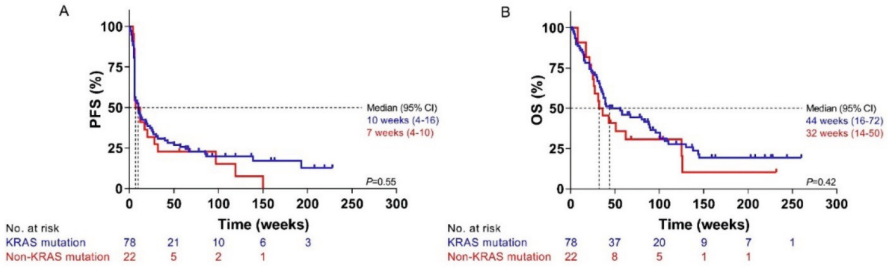
Supplementary Figure 5. Mutant ctDNA levels at baseline

(A) Number of mutant copies per mL of plasma measured prior to start of treatment for patients with no clinical response (<6 months) and durable clinical benefit (≥6 months). Red-colored results were ctDNA negative and these were excluded from the statistical analysis. These included both non-responders (21/63, 33%) and durable responders (10/37, 27. Kaplan-Meier plots displaying the (B) PFS and (C) OS by separating ctDNA levels at baseline in ctDNA low (below or equal to median levels, *n*=35), ctDNA high (above median, *n*=34) and ctDNA negative (*n*=31). Patients with low and high mutant ctDNA levels at baseline separated significantly with respect to PFS (*P*<0.001) and OS (*P*<0.0001). CI, confidence interval; PFS, progression-free survival; OS, overall survival.



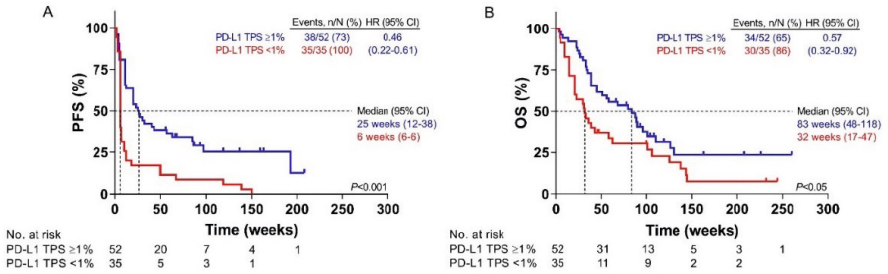
Supplementary Figure 6. PFS and OS at different cut-offs to determine ctDNA decrease

Progression-free survival (A-C) and overall survival (D-F) were analyzed at a 30% (A,D) 40% (B,E) and 50% (C,F) cut-off to determine ctDNA decrease. Increasing the cut-off resulted in slightly lower hazard ratios (HRs), however reduces the number of patients demonstrating a ctDNA decrease. The empirically determined technical cut-off of 30% results in highly significant hazard ratios (P<0.0001) and identifies the most patients with a durable response to ICI treatment.



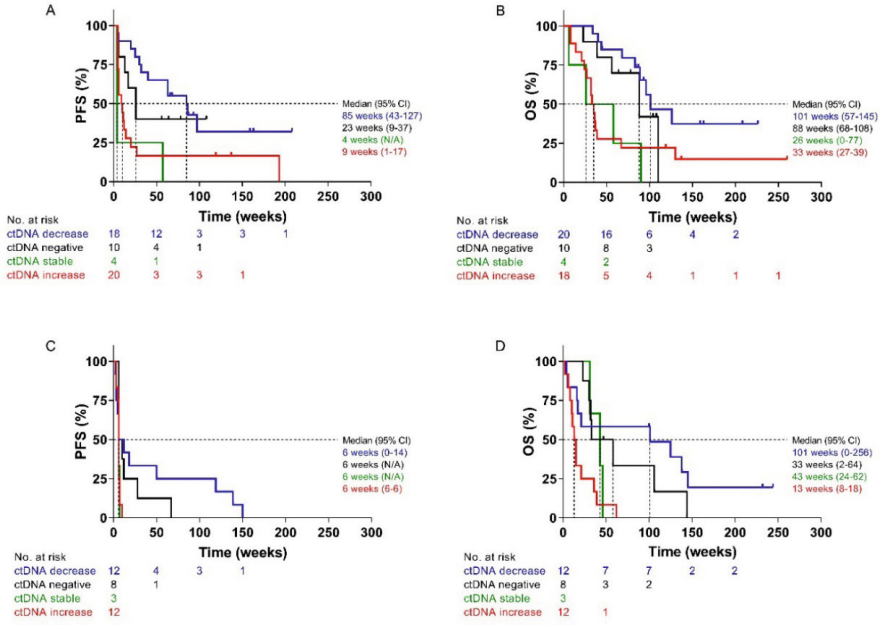
Supplementary Figure 7. PFS and OS is irrespective of *KRAS* mutations

Kaplan-Meier plot displaying the (A) PFS and (B) OS of patients harboring mutations in *KRAS* (blue), or in any other gene detected with ddPCR (red) ctDNA levels. CI: confidence interval; PFS: progression free survival; OS: overall survival.



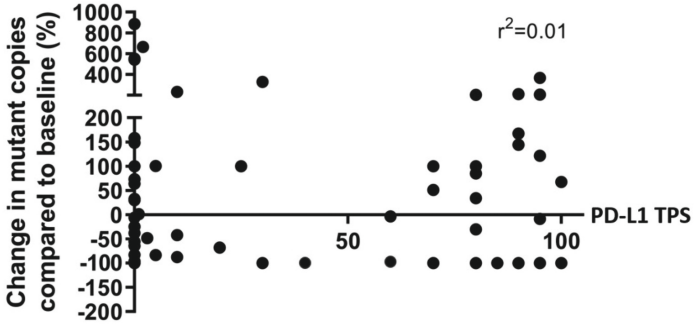
Supplementary Figure 8. Clinical response related to PD-L1 expression

Kaplan-Meier plots displaying the (A) PFS and (B) OS of patients with a tumor PD-L1 expression of <1% (red) or ≥1% (blue). CI: confidence interval; PFS: progression free survival; OS: overall survival.



Supplementary Figure 9. Elaborate analysis of radiological response related to PD-L1 expression

Extended representation of clinical response related to change in ctDNA levels in patients with a tumor PD-L1 TPS ≥1% (A-B) and TPS<1% (C-D). Kaplan-Meier plot displaying the (A, C) PFS and (B, D) OS of patients with decreasing (blue), negative (black), stable (green), or increasing (red) ctDNA levels. CI: confidence interval; PFS: progression free survival; OS: overall survival; TPS: Tumor Proportion Score.



Supplementary Figure 10. No correlation between change in ctDNA levels and PD-L1 TPS.

Pearson's correlation coefficient, r^2=0.01 is not considered significant.

Chapter 7

Response to immune checkpoint inhibition is associated with the gut microbiome in advanced *K-ras* mutated non-small cell lung cancer

Birgitta I. Hiddinga*
Laura A. Bolte*
Paul van der Leest
Lucie B.M. Hijmering-Kappelle
Anthonie J. van der Wekken
Ed Schuuring
Ranko Gacesa
Geke A.P. Hospers
Rinse K. Weersma
Johannes R. Björk
T. Jeroen N. Hiltermann

*contributed equally
In preparation for submission

ABSTRACT

Background

K-ras mutated non-small cell lung cancer (NSCLC) is associated with a poor prognosis to standard therapies. Despite advances of immune checkpoint inhibitors (ICIs), not all patients show durable responses. In this study, we aim to identify associations between ICI-response and the gut microbiome in patients with K-ras mutated NSCLC.

Methods

We performed shotgun metagenomic sequencing of stool samples collected before ICI initiation from 33 patients with K-ras mutated NSCLC. Microbiome composition within (α-diversity) and between samples (β-diversity) was calculated using Shannon diversity index and principal component analysis on Aitchison distances, respectively. A Bayesian logistic-normal regression model (Pibble) was implemented to identify associations between gut microbial features and disease control rate (DCR), progression free survival at 12 months (PFS12) and immune related adverse events (irAEs), adjusting for ICI-regimen, metastatic disease stage, age, gender and BMI.

Results

Responders were enriched with several saccharolytic species, including Agathobaculum butyriciproducens, Fusicatenibacter saccharivorans, Bifidobacterium longum and Eubacterium ramulus. Non-responders harbored higher abundances of several Bacteroides and Blautia species. Patients unaffected by irAEs demonstrated higher abundances of biotin and butyrate synthesis pathways. Development of irAEs was associated with higher Alistipes finegoldii, Bifidobacterium longum and Bacteroides uniformis abundance. No differences were observed between responders and non-responders in Shannon diversity index (P=0.69) and overall microbial composition (P=0.82).

Conclusions

We show gut microbial species and pathways that are differentially abundant between responders and non-responders to ICI in the setting of K-ras mutated NSCLC. We find overlap with microbial signatures of response to ICI in other tumor types, potentially reflecting tumor-independent microbial mechanisms.

Box 1. Glossary

α-diversity: Quantifies the number of microbial species within each sample. To test for differences in α-diversity, we computed the Shannon diversity index.
Shannon diversity: A measure of α-diversity which penalizes rare species.
β-diversity*: Quantifies the similarity/dissimilarity between two different samples. To test for differences in β*-diversity, we performed a Principal Component Analysis (PCA) on clr-transformed relative abundances.
Aitchison distance: The euclidean distance between samples calculated on species relative abundances after center log-ratio transformation (clr). This distance is considered the gold standard for high throughput sequencing data.
Principal Component Analysis (PCA): A dimensionality reduction *technique that is used to reduce the size of a large dataset into a smaller one that keeps most of the information. The result of this analysis is typically visualized in an ordination plot.*
Bayesian logistic-normal regression model: Statistical models that allow associating compositional and overdispersed high throughput sequencing data (such as microbiome data) with covariates. In the *Pibble* model, regression coefficients are ranked to determine which microbial features changes the most between cases and controls with statistical significance achieved through Bayesian inference. Importantly, the rankings produced from relative abundances are identical to the rankings produced by absolute abundances.
Permutational multivariate analysis of variance (PERMANOVA): Non-parametric multivariate statistical permutation test. Distance-based method to test which variables could significantly explain interindividual variation in the gut microbiome composition. The test statistics directly use the distance matrix to partition β-diversity into different sources of variation.
Microbial species: Groups of microorganisms that share common genetic and phenotypic characteristics. A species is a group of similar organisms (strains) within a genus. Microbial species can play important roles in various biochemical pathways and metabolic processes, such as the breakdown of fiber through fermentation, which can produce energy and metabolic byproducts, short-chain fatty acids.
Microbial pathways: Refer to specific biochemical processes and metabolic pathways that are carried out by microorganisms. Predicted metabolic functions of gut microbiota are based on their annotated genome and can be captured by whole shutgun metagenomics sequencing.

INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide. Immune checkpoint inhibition (ICI) has demonstrated significant benefit for patients with advanced non-small cell lung cancer (NSCLC). Recently, pembrolizumab has moved forward as standard of care first line treatment, in NSCLC patients having a PD-L1 tumor proportion score (TPS) > 50% [1]. Nivolumab is standard of care for second and further line treatment of immunotherapy-naïve patients [2]. The Kirsten rat sarcoma viral oncogene homolog (*K-ras*) mutation is the most frequent genetic alteration found in NSCLC. *K-ras* mutations are associated with considerable heterogeneity in clinical characteristics and a poor prognosis to standard NSCLC therapies [3]. Immunotherapy seems to be an effective choice in patients with *K-ras* mutation, in any line of treatment and with better outcomes than chemotherapy [4]. However, responses in most patients are still poor and for up to 80% this treatment will have no favorable effect in terms of long-term survival [5].

The gut microbiome has been recognized as a hallmark of cancer [6]. Mechanisms through which the gut microbiome affects cancer development and progression include eliciting (innate) tumor promoting inflammation as well as escaping (adaptive) immune destruction [6, 7]. Moreover, the gut microbiome has been linked to ICI response, including the development of immune-related adverse events (irAEs), suggesting that characterization of the gut microbiome may enable a more personalized line of treatment [8, 9, 10]. While most of the evidence comes from melanoma patients, it is not yet clear whether the gut microbiome can serve as a target in patients treated with ICI for different tumor entities such as *K-ras* mutated NSCLC [11, 12]. In a French cohort of 338 patients with NSCLC, of which about 40% *K-ras* mutated NSCLC, baseline *Akkermansia muciniphila* abundance was associated with increased response rates and overall survival [13].

In this study we investigate the role of the gut microbiome in a cohort of patients treated with anti-PD-1 immunotherapy for advanced *K-ras* mutated NSCLC, presenting a specific tumor entity that has not yet been studied in this setting.

MATERIALS AND METHODS

Participant selection

From 1st of October 2017 to 1st of December 2019 we enrolled 40 patients with stage IIIB to IVB *K-ras* mutated NSCLC (TNM classification of lung cancer, 8th edition) for treatment with ICI as first line treatment (14 patients pembrolizumab, PD-L1 TPS >50%) or after failing first line platinum containing doublet chemotherapy (26 patients nivolumab,

independent of PD-L1 score). Patients were treated with nivolumab 3 mg/kg every 2 weeks or pembrolizumab 200 mg flat dose every 3 weeks until progression or intolerable toxicity.

All patients were selected from a cohort of NSCLC patients harboring a *K-ras* mutation, as reported previously [14]. Key eligibility criteria are depicted in Supplementary table 1. Baseline characteristics, tumor stage and previous treatment are presented in Table 1. Antibiotic and proton pump inhibitor (PPI) use within 3 months of commencing ICI were documented.

Of 40 recruited participants two were excluded due to presence of a second primary tumor and not harboring a *K-ras* mutation, respectively. Five participants did not collect a fecal sample, leading to 33 patients eligible for analysis.

Sample collection, DNA extraction and sequencing

Patients received oral and written instructions about the stool sample collection. Stool samples were collected at baseline. The protocol for fecal sample collection and profiling of gut microbiota was previously published [15]. Microbial DNA was isolated with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. Metagenomic sequencing was performed at Novogene, China using the Illumina HiSeq 2000 platform. We obtained a total of 7.9 (sd=1.2) Gb with an average of 26.3 (sd=4.0) mil. reads/sample prior to quality control and pre-processing.

Sample processing

Reads aligning to the human genome (GRCh37/Hg19) were removed using KneadData integrated Bowtie2 tool (V.2.3.4.1), functional profiles were calculated using *HUMAnN3* (V.0.10.0) and the taxonomic composition was evaluated using *MetaPhlAn3*. Microbes and microbial functions that were present in less than 10% of samples and microbes with a relative abundance lower than 0.01% were not included in subsequent analyses. Samples with a sequencing depth below 10 million reads were removed. Arcsine square-root transformations for taxonomic abundances and logarithmic transformation for pathways were used as normalization methods.

Radiological evaluation and definition of clinical endpoints

Radiological evaluation with CT-scan according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 [16] was performed at baseline and every 6 weeks in the first year of ICI treatment, and thereafter every 12 weeks until disease progression.

Clinical endpoints were disease control rate (DCR), progression-free survival at 12 months (PFS12) and development of immune-related adverse events (irAEs).

DCR was defined on the basis of the first two radiological evaluations (at week 6 and week 12) using RECIST v1.1 criteria [16], classifying patients as responders (complete response, partial response, or stable disease) or non-responders (progressive disease). Stable disease was only classified as a response when confirmed at 6 months.

PFS was defined as the time from the first dose of an ICI to the first event; i.e., disease progression or death from any cause, with PFS12 indicating a complete/partial response or disease stability up to at least 12 months following initiation of ICI treatment.

IrAEs during or after ICI treatment were documented using the Common Terminology Criteria for Adverse Events (CTCAE) v5 (Table 1) [17]. Side effects of clearly non-immune etiology were excluded.

Statistical analysis

The statistical tests and terminology are described in Box 1. χ2 Tests for categorical variables and Mann-Whitney U test (MWU) for continuous data were performed to calculate differences between responders and non-responders (Supplementary Table 2). To test for differences in α-diversity, we computed Shannon diversity index using estimate richness (..., measures="Shannon") from the phyloseq package [18]. To test for differences in β-diversity, we performed a Principal Component Analysis (PCA) on clr-transformed relative abundances using transform(..., transform="clr") and ordinate(..., method="RDA") from the microbiome and phyloseq package, respectively [18]. To determine which endpoints and variables could significantly explain interindividual variation in the gut microbiome in this cohort, we performed Permutational Multivariate Analysis of Variance (PERMANOVA) on an Aitchison distance matrix produced from species-level clr-transformed relative abundances using the function adonis from the vegan R package (v2.5-7) [19]. The P and R^2 values were determined by 9999 permutations using all variables in the model.

To identify associations between treatment outcomes and species abundance and metabolic pathways, we implemented a Bayesian logistic-normal linear regression model called *Pibble* from the R package fido [20, 21], which allows for associating covariates to compositional and over dispersed high throughput sequencing data (Box 1).

We were particularly interested in the covariates determining whether a likely association existed between the gut microbiome and response to ICI, either DCR (yes/

no) or PFS12 (yes/no), ≥ grade 2 irAEs (yes/no), ICI regimen (Nivolumab/Pembrolizumab), and metastatic disease stage (1A, 1B, 1C), also adjusting for age, gender and BMI. Prior to fitting the model, we mean-centered the continuous covariates age and BMI. Furthermore, we used weighted sum/deviation coding (as opposed to treatment coding) which effectively mean-centers categorical covariates, to weight cases and controls by the number of observations [22]. Then, from the fitted model, we calculated the difference in the marginal means for cases vs. controls for each covariate of interest, and then ranked those to determine which microbial features changed the most between cases and controls. We report results at 75% and 90% credible intervals. This means we concluded that a microbial species or pathway is differentially abundant between cases and controls if 75% or 90% of its posterior distribution do not contain zero (i.e. 75% and 90% Bayesian Confidence Level, BCL).

RESULTS

Patient characteristics

Clinical and pathological characteristics are summarized in Table 1. The median PFS was 1 month (min=0 months, max=51 months, censoring date August 4, 2022), DCR was 24% (8 patients) and PFS12 was 18% (6 patients). Concomitant PPI was 58% and antibiotic use was low (9%). The *K-ras* G12C mutation was most frequently found (49%). In 11 patients (33%) irAEs occurred, of which 10 were ≥ CTCAE-grade 2. In responders, grade and number of organs affected by irAEs was higher than in non-responders, although not statistically significant (Supplementary table 2).

Table 1. Cohort characteristics

	(n=33)
Patient characteristics	
Age (years) at stage IV diagnosis, mean (SD)	64.24 (7.83)
Gender, n (%)	
Female	18 (55)
Male	15 (45)
BMI (kg/m^2), mean (SD)	24.93 (4.53)
Performance status, n (%)	
PS 0	6 (18)
PS 1	23 (70)
PS 2	4 (12)
Metastatic stage, n (%)	
0 (Stage 3b)	1 (3)
1a	10 (30)
1b	12 (36)
1c	10 (30)

Table 1. Continued.

	(n=33)
Treated brain metastases, n (%)	3 (9)
Liver metastases, n (%)	3 (9)
Smoking, n (%)	
Current smoker	4 (12)
Former smoker	29 (88)
Treatment characteristics	
ICI used, n (%)	
Nivolumab (second or further line)	21 (64)
Pembrolizumab (first line)	12 (36)
Antibiotic use at baseline, n (%)	3 (9)
PPI use at baseline, n (%)	19 (58)
Outcomes following ICI	
DCR, n (%)	8 (24)
PFS (months), median (range)	1.0 (0-51)
PFS12, n (%)	6 (18)
irAEs, n (%)	11 (33)
Maximum grade irAEs, n (%)	
0	22 (67)
1	1 (3)
2	3 (9)
3	4 (12)
4	3 (9)

Cohort characteristics are presented as mean and standard deviation (SD) for continuous variables and as counts and percentages for categorical variables. Abbreviations: BMI: body mass index; DCR: Disease control rate; ICI: immune checkpoint inhibitor; irAEs: immune-related adverse events; NSCLC: Non-small cell lung cancer; PFS: Progression free survival; PFS12: Progression free survival at 12 months; PPI: proton pump inhibitors.

Overall gut microbiome composition and diversity

We tested whether the gut microbiome of responders and non-responders exhibited differences in α-diversity and β-diversity and we found no difference between responders and non-responders in the Shannon diversity index neither for microbial species nor pathways in the PFS12 group (Figure 1). Similarly, there was no difference between responders and non-responders when using DCR as response measure (both *P*=0.69; Supplementary figures 1-2).

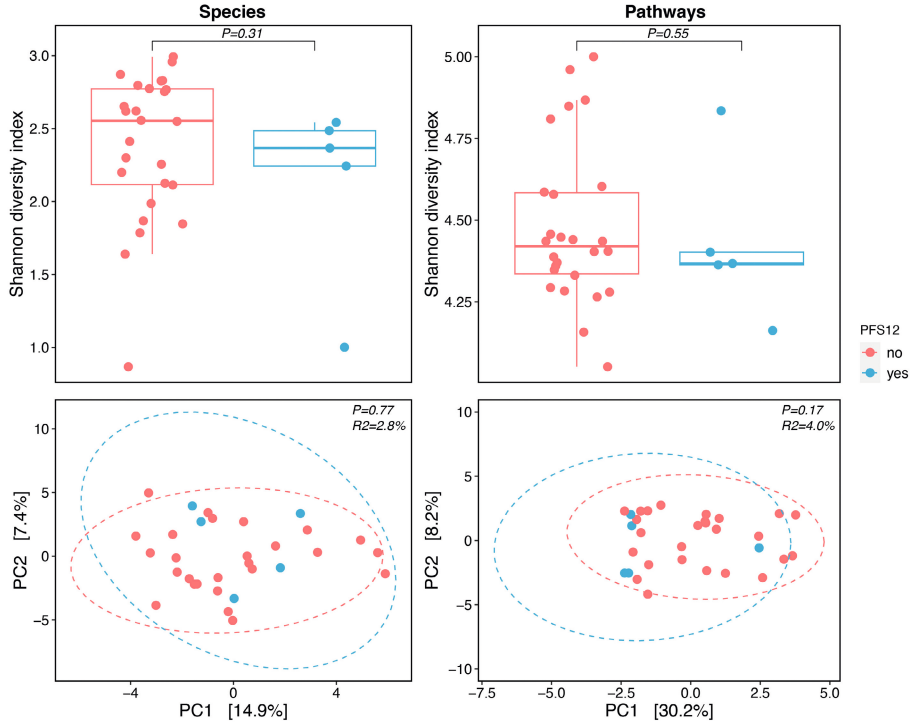


Figure 1: α-and β-diversity between responders and non-responders. Upper panels show α-diversity at the species- (left) and pathway-level (right).

α-diversity is computed as the Shannon diversity index (y-axis) for responders (R; blue) and non-responders (NR; red), respectively. Lower panels show species-and pathway-level compositional similarity (β-diversity) between responder and non-responder samples. β-diversity was computed using Aitchison distances. Each eclipse includes 95% of each group's samples.

We did not find significant differences in microbial species or pathway composition between responders and non-responders for either PFS12 nor DCR (Supplementary table 3). Thereafter, we tested whether patients who developed irAEs exhibited differences in α-and β-diversity compared to those who were resistant to irAEs. Similarly to response to ICI, we found no differences between these two patient groups in terms of the Shannon diversity index for species, pathways, nor for microbial species or pathway composition

(Supplementary figures 1-2, Supplementary table 3). In the PERMANOVAs, we also included and tested whether ICI regimen, metastatic disease stage, gender, age and BMI explained variation in gut microbiome composition. For species composition, we found that metastatic disease stage, and gender were the variables explaining most variation in both the DCR and PFS12 model. For microbial pathway composition we found that response (DCR and PFS12), ICI regimen, metastatic disease stage, and age explained the largest percent variation (between 4 and 6%). However, none of the variables reached statistical significance (Supplementary table 3).

Differential abundance analysis

Responders show enrichment of short chain fatty acids (SCFA)-producers

Responders were enriched in several saccharolytic species involved in the synthesis of short chain fatty acids (SCFA), including *Agathobaculum butyriciproducens*, *Clostridium leptum, Bifidobacterium longum*, *Eubacterium ramulus* and *Fusicatenibacter saccharivorans* (Figure 2A). Furthermore, responders showed higher relative abundances of *Akkermansia muciniphila* and SCFA-producers *Alistipes putredinis* and *Alistipes finegoldii* compared to non-responders, although these associations showed a wider credible interval (Figure 2). At pathway level, responders showed a higher abundance of pathways involved in synthesis of biotin (BIOTIN-BIOSYNTHESIS-PWY) and butyrate (PWY-5676; PWY-5022) (Figure 2B).

Responders show higher abundance of immunogenic pathways

In contrast to the aforementioned health-associated microbial features, we also observed that responders had higher abundances of pathways involved in lipopolysaccharide (LPS) and heme synthesis (NAGLIPASYN; PWY-5136; FAO-PWY; HEMESYN2-PWY, Figure 2B). These pathways are generally regarded as pro-inflammatory.

Higher abundance of Bacteroides species in non-responders

Non-responders were enriched in several species belonging to the Bacteroides (*Bacteroides (B.) sp CAF 44; B. clarus; B.wexlerae; B. uniformis; B. plebeius; B. ovatus*) and Blautia genus (*Ruminococcus gnavus; Blautia sp. CAG 257*) in both the DCR and the PFS12 model. Non-response was also associated with a higher abundance of *Escherichia coli* and several amino acid synthesis pathways (Figure 2).

Biotin and SCFA synthesis pathways enriched in patients unaffected by irAEs

Patients who did not develop irAEs showed higher abundances of *Anaerotruncus colihominis* and *Hungatella hathewayi* (that were associated with non-response) and *Anaerostipes hadrus* (Supplementary figure 3). These patients further showed an

enrichment of pathways involved in the synthesis of biotin and SCFA or precursors of SCFA (BIOTIN-BIOSYNTHESIS-PWY; PWY-5676; PWY-5022) and fatty acid synthesis (FASYN-INITIAL-PWY; FASYN-ELONG-PWY); Supplementary figure 4).

On the other hand, patients who developed irAEs exhibited higher abundances of *Alistipes finegoldii* and *Bifidobacterium longum* (that were enriched in responders) and *Bacteroides uniformis* (enriched in non-responders; Supplementary figure 3). Development of irAEs was associated with microbial synthesis of several amino acids (lysine, arginine, proline, isoleucine, asparagine; Supplementary figure 4) partially overlapping with pathways seen enriched in non-responders (Figure 2B).

Comparison of different ICI-regimen and metastatic disease stages

Finally, we compared gut microbial abundances in different treatment settings, including metastatic disease stage and treatment line or agent. We observed higher abundances of *Bacteroides sp. CAG 144* and *Escherichia coli* in those treated with first-line pembrolizumab compared to those treated with second- and further line nivolumab (Supplementary Figure 3). Patients with an earlier metastatic disease stage (M1a) harbored higher abundances of *Bifidobacterium* and *Eubacterium spp*., biotin synthesis (BIOTIN-BIOSYNTHESIS-PWY) and starch degradation (PWY-2723) pathways, whereas the higher metastatic disease stage M1c was associated with higher abundances of a pathway involved in LPS synthesis (NAGLIPASYN-PWY; Supplementary Figures 5-6).

DISCUSSION

In this study we profiled the gut microbiome composition and function in a homogeneous cohort of patients treated with anti-PD-1 immunotherapy for advanced *K-ras* mutated NSCLC. We identified distinct gut microbial features of response and non-response to treatment, while correcting for important clinical confounders such as ICI-type, metastatic disease stage and the development of irAEs. In line with earlier studies, response-associated microbiome features were not reflected at the whole microbiome level by common β-diversity metrices [23].

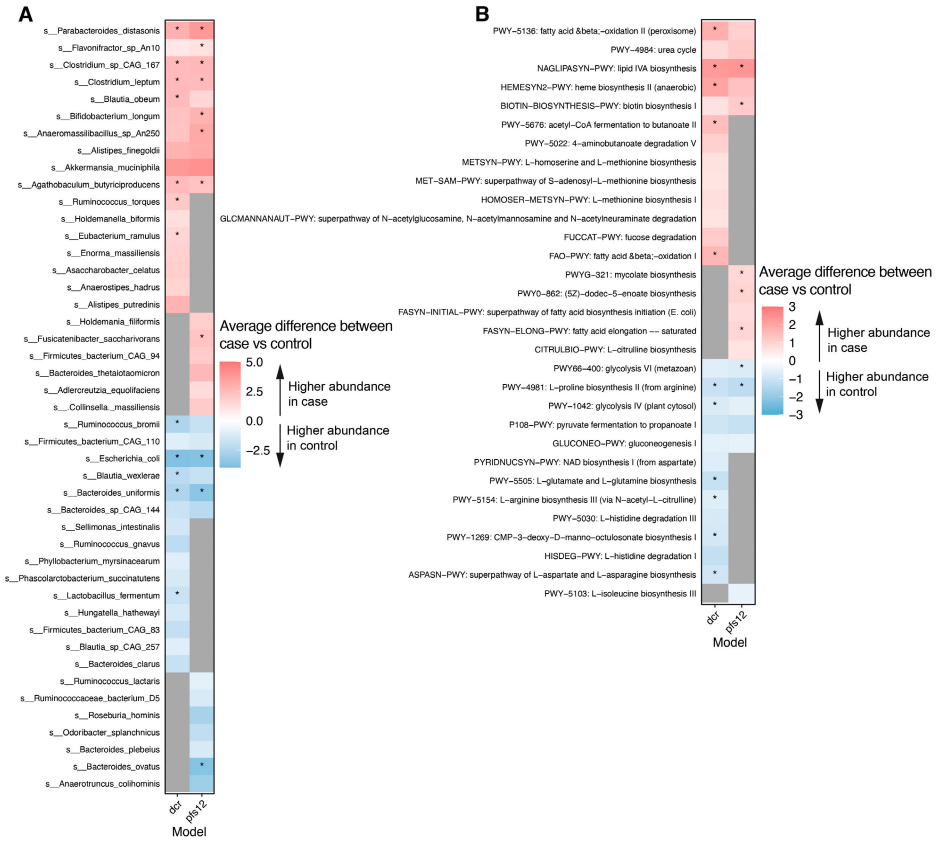


Figure 2: Differential abundance analysis

Differentially abundant microbial species (A) and pathways (B) between responders (R; red) and non-responders (NR; blue) at 75% Bayesian confidence level. Dots indicate microbial features that were differentially abundant at 90% Bayesian confidence level. Color strength indicates the effect size.

Overlap in microbial signatures of response with other tumor types and geographies

We found that responders are enriched in SCFA producing species compared to non-responders. SCFA producing species have previously been associated with healthy host phenotypes [11, 15, 24, 25]. SCFA-producers such as *Bifidobacteria* [23, 10], *Faecalibacterium prausnitzii* [9, 26] have been consistently associated with ICI-response across several tumor types including melanoma and renal cell carcinoma [8, 27], geographies [23, 28], and treatment regimen [13, 29].

Similarly, *Akkermansia muciniphila* has been repeatedly associated with increased overall survival and response rates to ICI in cohorts from different countries, such as in advanced

NSCLC patients from France [8, 13] and Poland [30], and melanoma patients from the Netherlands [31], the UK and Spain [23]. In our cohort we found increased *Akkermansia muciniphila* in responders, but not statistically significant, probably due to the small sample size. We also identified two *Alistipes* species to be enriched in responders, in line with the findings in melanoma and NSCLC patients [8, 26, 32].

At the level of predicted metabolic pathways, we observed an enrichment of biotin synthesis in responders as well as in patients unaffected by irAEs. Microbial pathways for the synthesis of biotin, as well as other B-vitamins, have been reported to be enriched in those protected from ICI-induced colitis [33].

Non-responders showed higher abundances of the species belonging to the Blautia genus and the Bacteroides genus. These species have been previously shown to be associated with chronic diseases such as IBD, diabetes mellitus and cardiovascular diseases and long-term diets rich in animal protein and saturated fat [15, 24, 25]. Our observation aligns with the findings of a recent cross-cancer meta-analysis at which *Bacteroides* were relatively underrepresented in ICI-responders across different tumor types including NSCLC, melanoma, hepatic and renal cell carcinoma [8]. *Bacteroides clarus* in particular, has been consistently associated with non-response to ICI [23].

Differences to NSCLC cohorts from other geographies

Overall, there has been heterogeneity in the microbial species associated with response across different cohorts [23], owing to regional differences such as diets, including concomitant medication use, and to methodological confounders such as limited sample sizes, metastatic disease stage or ICI-type not considered. In contrast to our findings, a study from China found higher abundances of Bacteroidea such as *Bacteroides massiliensis* in NSCLC patients who showed a partial response after anti-PD-1 therapy [32]. Interestingly, previous studies have shown biphasic effects for the *Bacteroides* genus: while the *Bacteroides* genus has been associated with negative efficacy of anti-PD-1 blockade, in line with our study [9], some *Bacteroides* species (*B. fragilis, B. thetaiotaomicron)* have been shown to increase efficacy when anti-CTLA-4 blockade was used [29]. In another study from China, NSCLC patients treated with nivolumab showed an enrichment of *Alistipes*, *Bifidobacterium* and *Prevotella*, whereas *Ruminococcaceae* was associated with non-response [34]. Two studies from Japan in ICI-treated NSCLC patients found yet another set of species associated with response, mainly *Ruminococcaceae* and *Agathobacter* [35] and *Lactobacillus*, *Clostridium* and *Syntrophococcus* [36], whereas non-responders were enriched in *Bilophila*, *Sutterella* and *Parabacteroides*. Another reason for the observed differences could be that, while the patients in our cohort all harbored a *K-ras* mutation, previous studies conducted

in NSCLC did not look at these patients separately. Larger studies across different geographies are needed to further elucidate the role of the gut microbiome in NSCLC.

Higher abundance of inflammatory pathways in responders

While previous studies suggest a health-associated gut microbiome profile in responders to ICI [37], including higher abundances of SCFA-producers, we also observed an enrichment of microbial functions that are generally considered "pro-inflammatory" or immunogenic. The precise mechanisms between the gut microbiome and immunotherapy still have to be elucidated [38]. Our findings suggest that different microbial mechanisms are at play that could help promote an anti-cancer immune response during ICI treatment, challenging the concept of predominantly healthy microbiome signatures associated with response. Given the role of SCFA producing species in the fermentation of fiber, our results support a potential benefit of fiber-rich diets and unsaturated fatty acids to improve outcomes of ICI therapy [39, 40].

STRENGTHS AND LIMITATIONS

To our knowledge this is the first study associating outcomes to ICI with the gut microbiome composition that is conducted exclusively in NSCLC patients harboring a KRAS mutation, a tumor entity that has been hard-to-target by standard NSCLC therapies in the past. A limitation of the study lies in its sample size and studying only a single time point (pre-treatment). Future multinational studies across different tumor entities with longitudinal profiling of the gut microbiome are needed to confirm the role of the identified species as potential biomarkers or treatment targets to improve response to ICI.

CONCLUSIONS

In a homogeneous cohort of patients with a *K-ras* mutated NSCLC, we identified microbial species and pathways associated with response to ICI, and the development of irAEs. We find overlap in gut microbial species and functions associated with ICI-response in other tumor types such as melanoma, that may reflect tumor-independent microbial mechanisms. Specifically, we identified an enrichment in species involved in the fermentation of fiber and production of SCFA in responders, supporting a potential benefit of fiber-rich diets to synergize with ICI. Non-responders harbored a higher abundance of species belonging to the *Bacteroides* genus. The findings support the notion that the gut microbiome could be an interesting target to improve outcomes in NSCLC patients treated with ICI.

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Supplementary Table 1. Key eligibility criteria

Inclusion criteria	Exclusion criteria
NSCLC stage IIIB to IVB	Active brain or leptomeningeal metastases
K-ras mutation	Previous anti-PD(L)-1 immunotherapy
Age >18 years	Previous treatment for ALK rearrangement, EGFR or BRAF mutation
Life expectancy >3 months	Active, known or suspected autoimmune disease, except for T1DM
PD-L1 TPS > 50% (if first line ICI)	Hypothyroidism only requiring hormone replacement
ECOG performance score 0-1	Skin disorders including vitiligo, psoriasis, or alopecia, not requiring systemic treatment.
At least 1 measurable lesion by RECIST v1.1	
Adequate bone marrow reserve and organ function	
Completion of any prior palliative radiotherapy at least 2 weeks prior to ICI start	

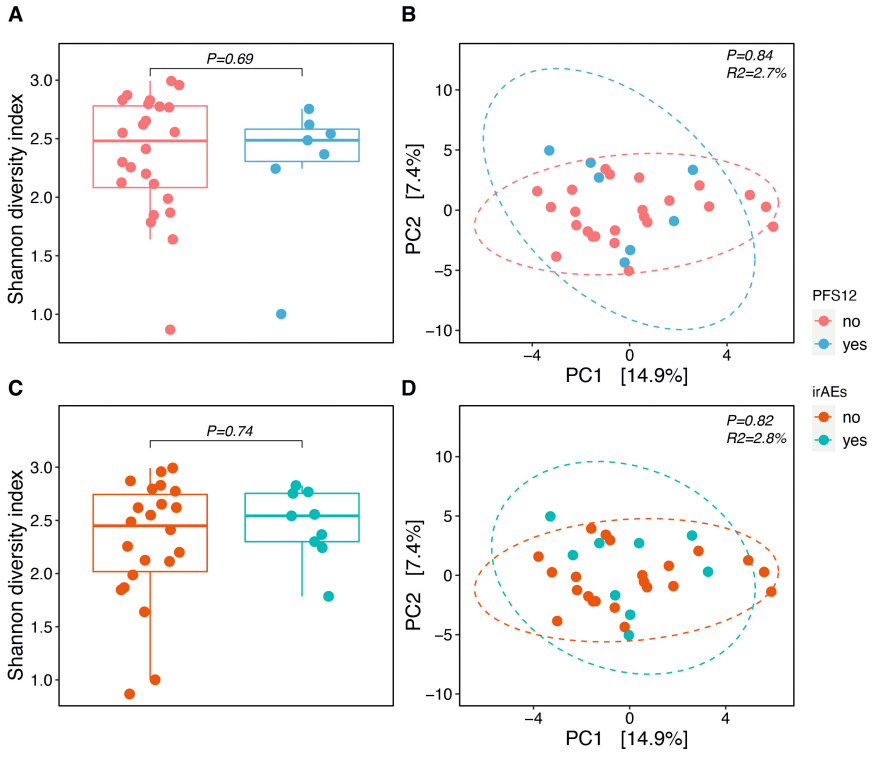
Abbreviations: Immune checkpoint inhibition, ICI; Eastern Cooperative Oncology Group, ECOG; Response Evaluation Criteria in Solid Tumors, RECIST; Type 1 diabetes mellitus, T1DM.

Supplementary Table 2. Descriptive statistics

	Total (n=33)	Responders (n=8)	Non-responders (n=25)	P-value
Patient characteristics				
Age (years) at stage IV diagnosis, mean (SD)	64.24 (7.83)	63.38 (8.23)	64.52 (7.86)	0.899
Gender, n (%)				0.081
Female	18 (55)	7 (88)	11 (44)	
Male	15 (45)	1 (13)	14 (56)	
BMI (kg/m^2), mean (SD)	24.93 (4.53)	24.45 (4.56)	25.08 (4.61)	0.867
Performance status, n (%)				1.000
PS 0	6 (18)	3 (38)	3 (12)	
PS 1	23 (70)	4 (15)	19 (76)	
PS2	4 (12)	1 (13)	3 (12)	
Metastatic stage, n (%)				0.182
0 (Stage 3b)	1 (3)	1 (13)	0 (0)	
1a	10 (30)	2 (25)	8 (32)	
1b	12 (36)	4 (50)	8 (32)	
1c	10 (30)	1 (13)	9 (36)	
Treated brain metastases, n (%)	3 (9)	0 (0)	3 (12)	0.748
Liver metastases, n (%)	3 (9)	0 (0)	3 (12)	0.748
K-ras mutation, n (%)				0.805
G12A	3 (9)	0 (0)	3 (12)	
G12C	16 (49)	4 (50)	12 (48)	
G12D	3 (9)	1 (13)	2 (8)	
G12S	1 (3)	0 (0)	1 (4)	
G12V	5 (15)	2 (25)	3 (12)	
G13C	1 (3)	0 (0)	1 (4)	
G13D	2 (6)	1 (13)	1 (4)	
Q22K	2 (6)	0 (0)	2 (8)	

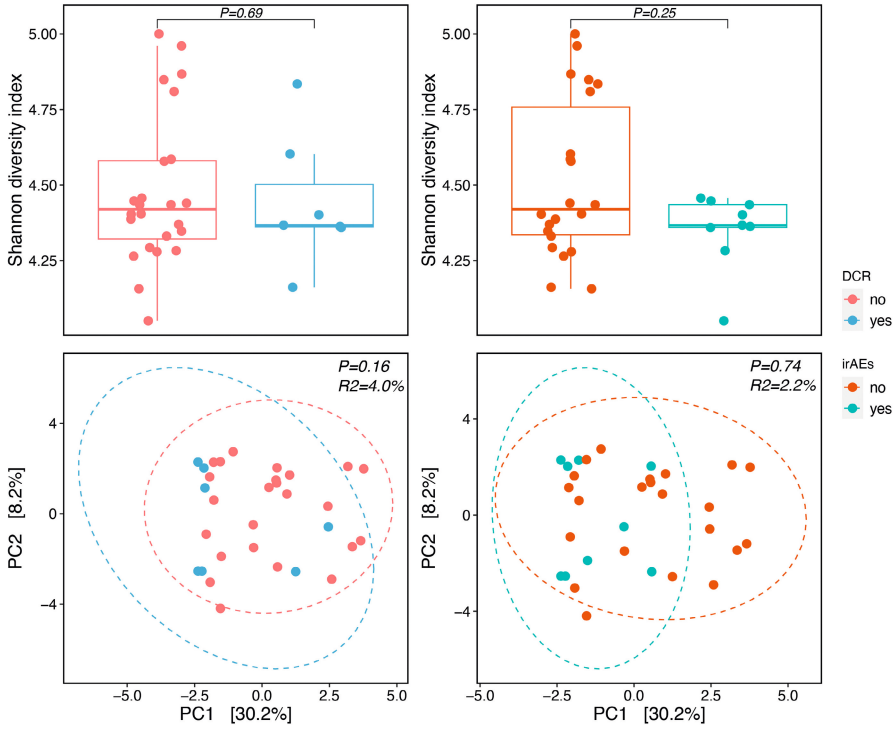
	Total (n=33)	**Responders (n=8)**	**Non-responders (n=25)**	**P-value**
Smoking, n (%)				1.000
Current smoker	4 (12)	1 (13)	3 (12)	
Former smoker	29 (88)	7 (88)	22 (88)	
Treatment characteristics				
ICI used, n (%)				0.179
Nivolumab (second or further line)	21 (64)	3 (38)	18 (72)	
Pembrolizumab (first line)	12 (36)	5 (63)	7 (28)	
Number of treatment lines prior to study, n (%)				0.068
0	12 (36)	5 (63)	7 (28)	
1	18 (55)	3 (38)	15 (60)	
2	3 (9)	0 (0)	3 (12)	
Antibiotic use at baseline, n (%)	3 (9)		3 (12)	0.748
PPI use at baseline, n (%)	19 (58)	4 (50)	15 (60)	0.931
irAEs, n (%)	11 (33)	5 (63)	6 (24)	0.114
Colitis, n (%)	3 (9)	1 (13)	2 (8)	1.0
Maximum grade irAEs, n (%)				0.042
0	22 (67)	3 (38)	19 (76)	
1	1 (3)	0 (0)	1 (4)	
2	3 (9)	2 (25)	1 (4)	
3	4 (12)	1 (13)	3 (12)	
4	3 (9)	2 (25)	1 (4)	
Number of organs affected by irAEs, n (%)				0.016
0	22 (67)	3 (38)	19 (76)	
1	6 (18)	1 (13)	5 (20)	
2	4 (12)	3 (38)	1 (4)	
3	1 (3)	1 (13)	0 (0)	

Baseline characteristics are presented as mean and standard deviation (SD) for continuous variables and as counts and percentages for categorical variables. Statistics are provided for the total cohort as well as the subset of responders and non-responders defined by DCR. $\chi 2$ tests for categorical variables and Mann-Whitney U test (MWU) for continuous data were performed to calculate differences between responders and non-responders. P-values written in bold indicate nominally significant differences between responders and non-responders ($P < .05$). There were no statistically significant differences between responders and non-responders after multiple hypothesis testing correction (FDR > 0.5). Abbreviations: BMI: body mass index; DCR: Disease control rate; FDR: False Discovery Rate; ICI: immune checkpoint inhibitor; irAEs: immune-related adverse events; PFS: Progression-free survival; PFS12: Progression-free survival at 12 months; PPI: proton pump inhibitors.



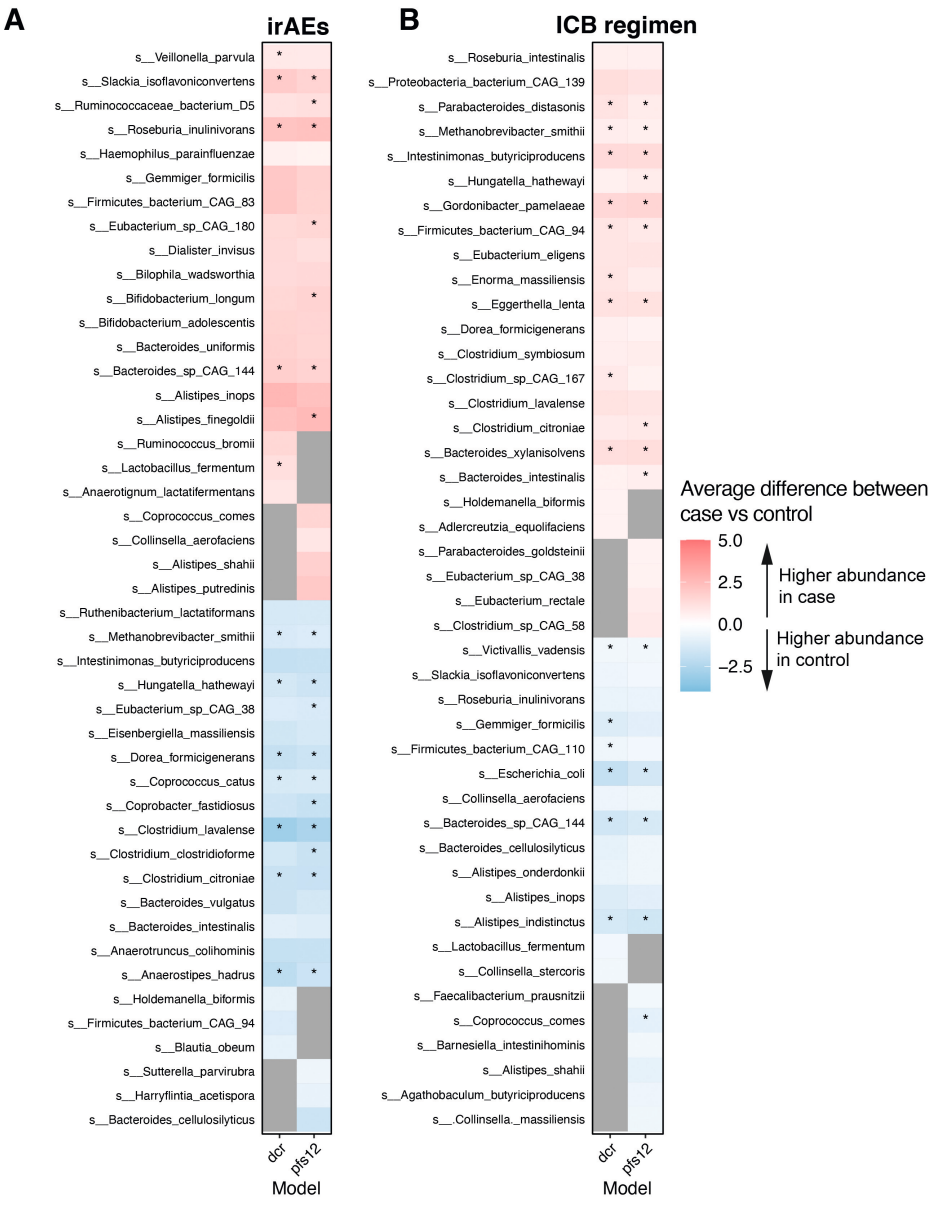
Supplementary figure 1. Alpha and beta diversity at the species level

Upper panels show α-diversity (left) and β-diversity (right) for responders (R; blue) and non-responders (NR; red) defined by disease control rate (DCR). α-diversity is computed as the Shannon diversity index (y-axis). Species-level compositional similarity (β-diversity) was computed using Aitchison distances. Each eclipse includes 95% of each group's samples. Lower panels show α-diversity (left) and β-diversity (right) for patients who developed immune-related adverse events during treatment (irAEs; blue) and those who did no (No irAEs; red).



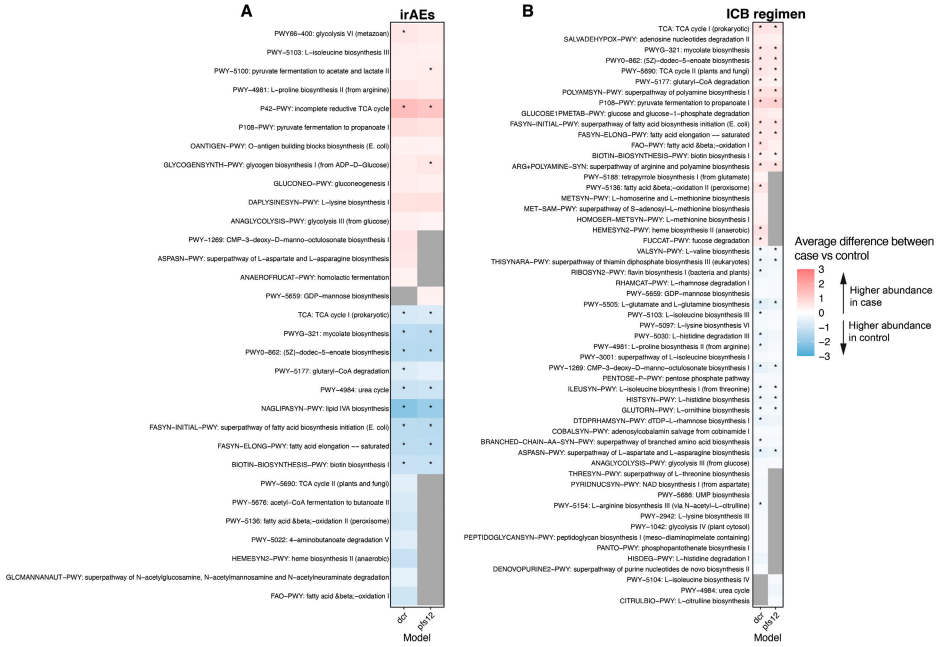
Supplementary figure 2. Alpha and beta diversity at the pathway-level

Upper panels show α-diversity (left) and β-diversity (right) for responders (R; blue) and non-responders (NR; red) defined by disease control rate (DCR). α-diversity is computed as the Shannon diversity index (y-axis). Compositional similarity (β-diversity) at the pathway-level was computed using Aitchison distances. Each eclipse includes 95% of each group's samples. Lower panels show α-diversity (left) and β-diversity (right) for patients who developed immune-related adverse events during treatment (irAEs; blue) and those who did no (No irAEs; red).



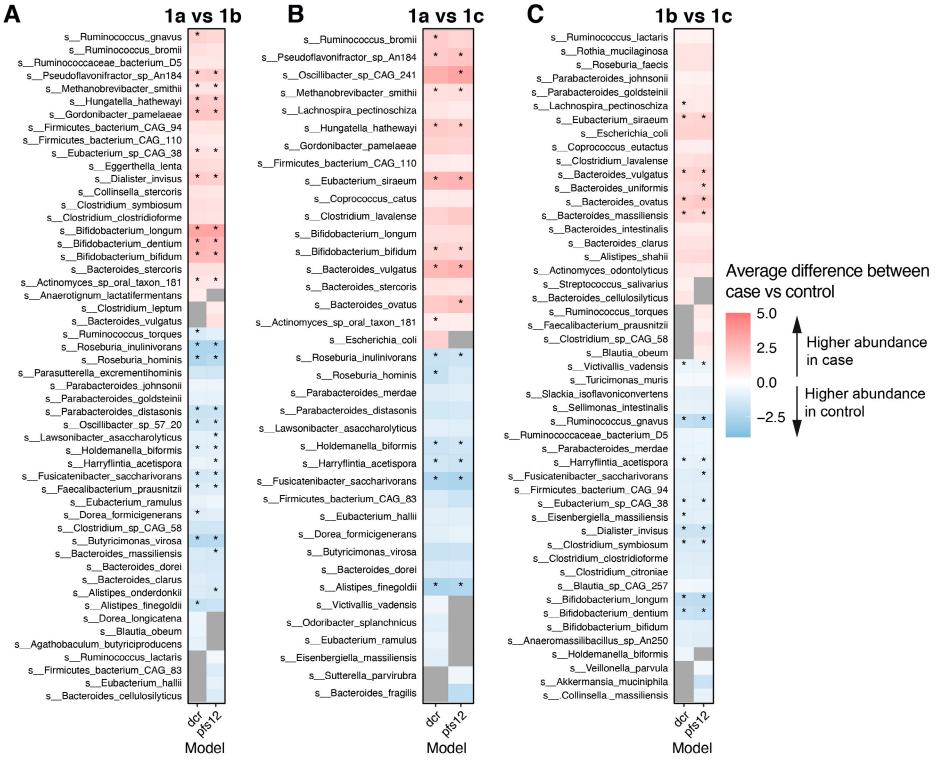
Supplementary figure 3. Species-level comparison of irAEs and different ICI-regimen

Left panel shows differentially abundant microbial species between patients developing irAEs (red) vs. no irAEs (blue) at 75% BCL. Right panel shows differentially abundant species between patients receiving second and further line Nivolumab (red) vs. patients receiving first line Pembrolizumab treatment (blue). Dots indicate microbial features that were differentially abundant at 90% BCL. Color strength indicates the effect size.



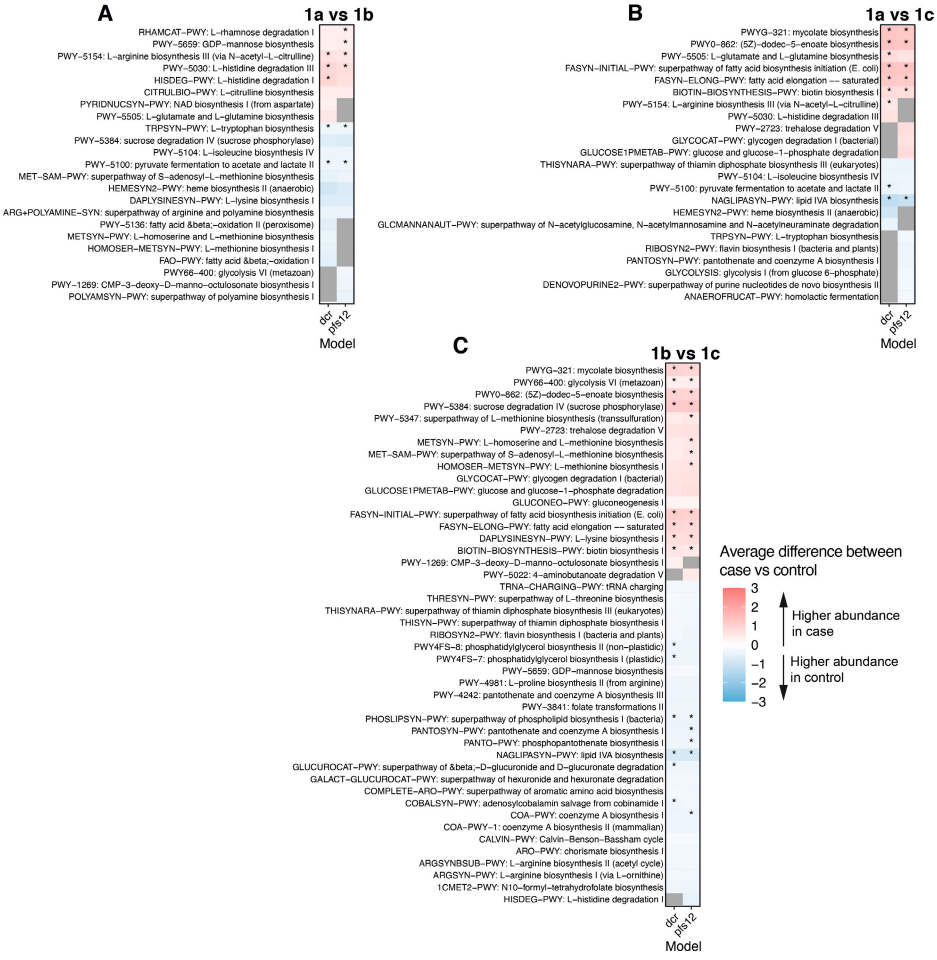
Supplementary Figure 4. Pathway-level comparison of irAEs and different ICI-regimen

Left panel shows differentially abundant microbial pathways between patients developing irAEs (red) vs. no irAEs (blue) at 75% BCL. Right panel shows differentially abundant pathways between patients receiving second and further line Nivolumab (red) vs. patients receiving first line Pembrolizumab treatment (blue). Dots indicate microbial features that were differentially abundant at 90% BCL. Color strength indicates the effect size.



Supplementary Figure 5. Species-level comparison of different metastatic disease stages

Differentially abundant microbial species between patients with early disease stages (red) compared to later disease stages (blue) at 75% BCL. Dots indicate microbial features that were differentially abundant at 90% BCL. Color strength indicates the effect size.



Supplementary Figure 6. Pathway-level comparison of different metastatic disease stages

Differentially abundant microbial pathways between patients with early disease stages (red) compared to later disease stages (blue) at 75% BCL. Dots indicate microbial features that were differentially abundant at 90% BCL. Color strength indicates the effect size.

Chapter 8

Lesson of the Month
Non-small-cell lung cancer infiltrated with chronic myelomonocytic leukemia: a molecular diagnostic challenge to recognize mixed cancers in a single biopsy

Bart Koopman
Birgitta I Hiddinga
Inge Platteel
Joost L Kluiver
Wim Timens
André B Mulder
Jaap A van Doesum
Ed Schuuring
Arjan Diepstra
Léon C van Kempen

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INTRODUCTION

Molecular profiling techniques such as targeted next generation sequencing (NGS) have become increasingly important in routine cancer diagnostics. Genomic alterations that are characteristic in certain malignancies are sometimes also detected in other cancers. Detection of rare variants may challenge the initial diagnosis or uncover a co-existing malignancy [1,2]. We report on a non-small-cell lung cancer (NSCLC) case with an oncogenic mutation in *PIK3CA* and unusual mutations in both *MET* and *IDH2*, the last of which was shown to originate from tumor-infiltrating chronic myelomonocytic leukemia (CMML).

CASE PRESENTATION

An 80-year-old male presented with a mass in the right upper lobe with accompanying brain and bone lesions, suspicious of a stage IVB (cT4N0M1c) primary tumor of the lung. A histological needle biopsy of the pulmonary mass showed a solid and trabecular growing (non-small-cell) carcinoma in a minority of Alcian blue-positive intracytoplasmic vacuoles. Immunohistochemistry showed a diffuse strong nuclear staining for thyroid transcription factor 1 (TTF1), diffuse strong cytoplasmic staining for napsin-A and lack of staining for p40, leading to a conclusion of poorly differentiated adenocarcinoma. NGS revealed the presence of an oncogenic mutation in *PIK3CA* (NM_006218): c.3140A>G p.(His1047Arg) with 34% variant allele frequency (VAF). Additionally, two unexpected mutations were detected: an unknown frameshift mutation in *MET* exon 14 (NM_000245): c.2913_2914delinsT p.(Asp972Metfs*13) with 48% VAF and a mutation in *IDH2* (NM_002168): c.419G>A p.(Arg140Gln) with an almost fourfold lower VAF (13%). NanoString-targeted transcript analysis demonstrated *MET* exon 14 skipping transcripts. This mutational profile was intriguing, because of the unknown mutation in *MET* resulting in exon 14 skipping and the *IDH2* mutation which is only rarely found in NSCLC and almost exclusively limited to hematological malignancies [3]. The patient provided written informed consent for publication.

Review of the patient's health record revealed a history of CMML with an *IDH2* p.(Arg140Gln) mutation. The CMML had been regularly followed-up every 8–12 weeks prior to the diagnosis of NSCLC. Complete blood counts and white blood cell differentials had repeatedly demonstrated stable disease without indication of transformation to acute myeloid leukemia. Flow cytometry-based analysis on peripheral blood performed after the lung cancer diagnosis demonstrated 1% myeloblasts and 8% CD64 strong positive/CD300e-negative monoblasts/promonocytes. Considering the patient's age and lack of anemia, thrombocytopenia or transformation to acute myeloid leukemia,

there was no indication for treatment. Re-analysis by NGS of the bone marrow tissue previously used to diagnose CMML confirmed the presence of *IDH2* p.(Arg140Gln) and demonstrated wild-type *PIK3CA* and *MET*. Together, this suggested that *IDH2* mutation-positive leukemic cells were present in the lung adenocarcinoma biopsy.

Re-analysis of the histology did not clearly identify the presence of leukemia; however, leukemic cells in CMML can be virtually indistinguishable from normal monocytes by histology alone. Microscopic analysis showed a modest presence of inflammatory-like cells, with an interstitial distribution pattern in the stroma and in association with the tumor cells, closely resembling reactive inflammation as often seen in the context of NSCLC. Immunohistochemical staining revealed that these cells were consistently CD163-positive, most probably reflecting CMML cells (Figure 1). There were no sheets of CMML cells and there were no blastic plasmacytoid dendritic cells or plasmacytoid dendritic cell aggregates – which can sometimes be present in peripheral tissues involved with CMML – recognizable by histology and flow cytometry.

To demonstrate the presence of the *IDH2* mutation in the inflammatory-like component, macrodissection and mutation-specific digital droplet polymerase chain reaction (ddPCR; BioRad, Lunteren, the Netherlands) analysis of adenocarcinoma and adenocarcinoma-free stromal tissue was performed. Both the *PIK3CA* and *IDH2* mutations were detected in the dissected area containing both the TTF1-positive adenocarcinoma and CD163-positive inflammatory-like cells with allelic frequency of 44% and 8%, respectively. The *MET* mutation was not analyzed due to the challenging design of the ddPCR probe and primers. In contrast, only the *IDH2* p.(Arg140Gln) mutation with an allelic frequency of 39% was detected in the tumor-free component. These observations indicate that only the CD163-positive inflammatory-like cells carried the *IDH2* mutation.

The case was discussed by the Molecular Tumor Board (MTB) at the University Medical Center Groningen [4]. There was no indication to treat the CMML due to lack of clinical manifestations such as anemia or thrombopenia, nor suspicion of transformation to acute myeloid leukemia. Targeted *MET* inhibition was recommended for treating the NSCLC based on the detected *MET* exon 14 skipping. The MTB acknowledged the unknown effect of the *PIK3CA* mutation on MET inhibition but did not recommend dual inhibition, as the treatment efficacy of PI3K inhibitors in NSCLC is unknown. Crizotinib in compassionate use (250 mg twice daily) was initiated and resulted in a 39% radiological volume reduction (7.6–4.6 cm) of the primary tumor within 12 weeks (Figure 2). Despite clinical and radiological improvement, the patient developed an occlusion of the superior mesenteric artery with intestinal ischemia and succumbed to subsequent abdominal sepsis. The patient's family did not consent to post-mortem examination and the cause of the obstruction therefore remained unknown.

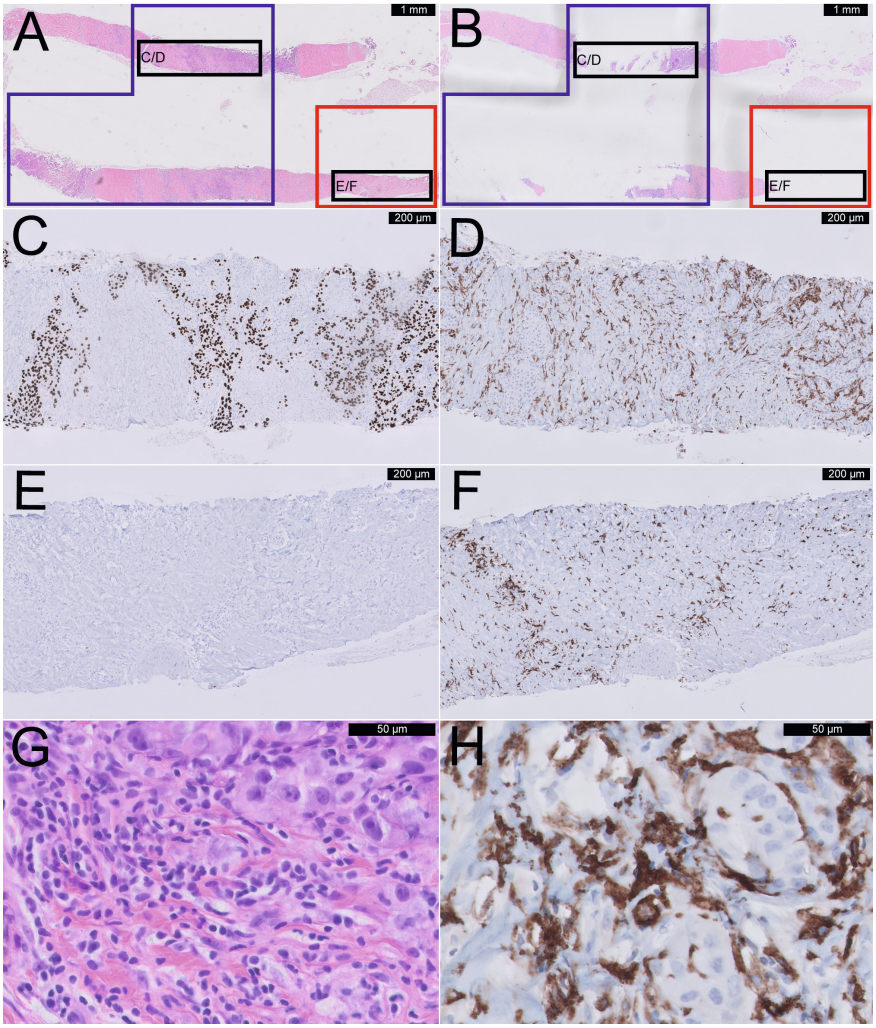

Figure 1. Histological images demonstrating the concurring presence of myelomonocytic leukemia cells and adenocarcinoma cells.

Histological images of the lung biopsy obtained by endobronchial ultrasound-guided fine needle aspiration, with corresponding TTF1 and CD163 immunohistochemistry. **A–B,** H&E stains of predissection (**A**) and post-dissection (**B**) tissue slides, with adenocarcinoma-containing, TTF1-positive and chronic myelomonocytic leukemia (CMML)-containing, CD163-positive areas marked in blue and tumor-free, TTF1-negative and CD163-positive areas marked in red. Part of the etching in the glass slide to mark the area for dissection is visible in **B**. **C–D,** Adenocarcinoma-containing, TTF1-positive (**C**) and CMML-containing, CD163-positive (**D**) area, in which both *PIK3CA* p.(H1047R) and *IDH2* p.(R140Q) were detected with mutation-specific ddPCR. **E–F,** Tumor-free, TTF1-negative (**E**) and CD163-positive (**F**) area, testing positive for *IDH2* p.(R140Q) but negative for *PIK3CA* p.(H1047R) with mutation-specific ddPCR. **G–H,** Area containing both adenocarcinoma (large cells, upper right) and suspected CMML cells (small cells, lower left), stained with H&E (**G**) and CD163 (**H**). *CD163, cluster of differentiation 163; ddPCR, digital-droplet polymerase chain reaction; H&E, hematoxylin and eosin; IDH2, isocitrate dehydrogenase [NADP(+)] 2; PIK3CA, phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha; TTF1, thyroid transcription factor 1.*

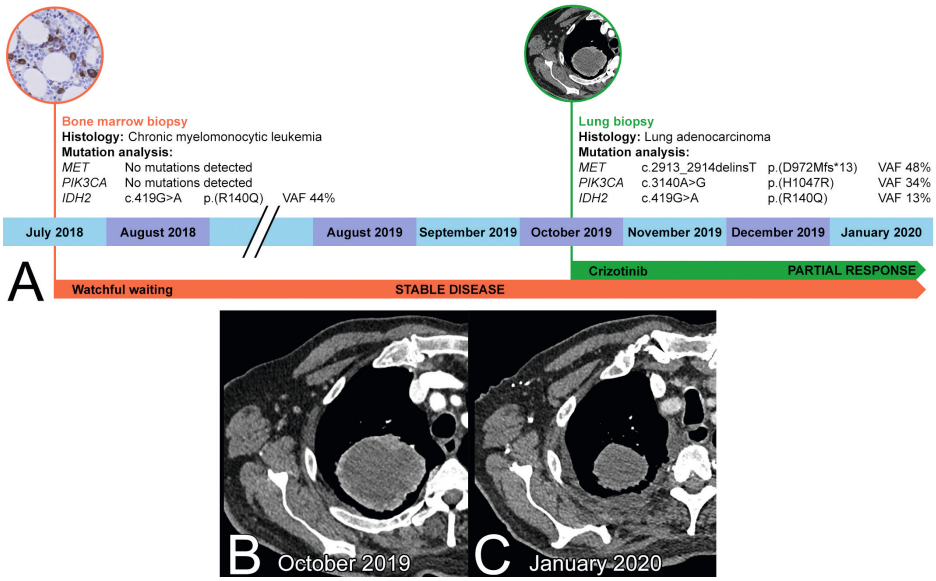

Figure 2. Patient clinical history and radiological imaging.

A, Timeline of the patient's clinical history, with respective NGS results marked for separate bone marrow and lung biopsies. B, Computed tomography imaging of primary lesion (maximum diameter 7.6 cm) in the right upper lobe of the lung prior to initiating treatment with crizotinib. C, Computed tomography imaging after three months of treatment with crizotinib showing a partial response, with a volume reduction of 39% (maximum diameter 4.6 cm). *IDH2, isocitrate dehydrogenase [NADP(+)] 2; MET, MET proto-oncogene, receptor tyrosine kinase; NGS, next-generation sequencing; PIK3CA, phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha.*

DISCUSSION

The two unexpected mutations found in this patient's biopsy each highlight unique diagnostic challenges derived from genomic profiling. The *IDH2* p.(Arg140Gln) mutation most probably originated from tumor-infiltrating CMML. Although uncovering a second primary malignancy based on genomic profiling is not uncommon [1,2], detection of two different types of cancer in a single biopsy is rare. Here the histological and molecular work-up of the case identified CMML-infiltrating lung adenocarcinoma.

In addition, we identified a novel uncharacterized *MET* exon 14 frameshift mutation p.(Asp972Metfs*13) which was shown to induce *MET* exon 14 skipping. Mutations in *MET* that involve the splice site acceptor or donor site of exon 14 result in the exclusion of exon 14 in the *MET* transcript with subsequent loss of the ability to down-regulate the signaling activity [5]. Evidence from CRISPR-Cas9-altered cell lines indicates that

frameshift mutations can also induce splicing events that result in exclusion of the affected exon from the transcript [6]. Although the exact mechanism remains elusive, expression of a *MET* exon 14 skipping RNA transcript was verified. The observed 39% volume reduction of the primary tumor after 12 weeks of targeted MET inhibition treatment demonstrated that this novel *MET* frameshift variant is indeed actionable.

This case illustrates that seemingly unexpected oncogenic mutations can be derived from a tumor infiltrating second malignancy that may be unrecognizable by histomorphology alone. This patient's NSCLC biopsy harbored an unexpected *IDH2* mutation which was shown to originate from tumor-infiltrating chronic myelomonocytic leukemia. We conclude that the presence of a second malignancy should be considered when an unexpected genetic variant is detected in the molecular analysis of solid malignancies.

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Chapter 9

The additional diagnostic value of virtual bronchoscopy navigation in patients with pulmonary nodules – The NAVIGATOR study

Birgitta I. Hiddinga
Dirk-Jan Slebos
T. David Koster
Lucie B.M. Hijmering-Kappelle
T. Jeroen N. Hiltermann
Hanneke Kievit
Anthonie J. van der Wekken
Gonda de Jonge
Rozemarijn Vliegenthart
Caroline Van De Wauwer
Wim Timens
Frederike Bensch

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ABSTRACT

Background

The number of solitary pulmonary nodules to be evaluated is expected to increase and therefore we need to improve diagnostic and therapeutic tools to approach these nodules. To prevent patients from futile invasive procedures and receiving treatment without histological confirmation of cancer, we evaluated the value of virtual bronchoscopy navigation to obtain a diagnosis of the solitary pulmonary nodule in a real-world clinical setting.

Methods

In the NAVIGATOR single center, prospective, observational cohort study patients underwent a virtual bronchoscopy navigation procedure with or without guide sheet tunnelling to assess a solitary pulmonary nodule. Nodules were considered not accessible if a diagnosis could not be obtained by either by CT-guided transthoracic biopsy or conventional bronchoscopy.

Results

Between February 2021 and January 2022 35 patients underwent the virtual bronchoscopy navigation procedure. The overall diagnostic yield was 77% and was dependent on size of the nodule and chosen path, with highest yield in lesions with an airway path. Adverse events were few and manageable.

Conclusion

Virtual bronchoscopy navigation with or without sheet tunnelling is a new technique with a good diagnostic yield, also in patients in whom previously performed procedures failed to establish a diagnosis and/or alternative procedures are considered not feasible based on expected yield and/or safety. Preventing futile or more invasive procedures like surgery or transthoracic punctures with a higher complication rate is beneficial for patients, and allowed treatment adaptation in two-third of the analyzed patient population.

HIGHLIGHTS

- Solitary pulmonary nodules to be evaluated is expected to increase over time.
- Virtual bronchoscopy navigation with or without sheet tunnelling has a diagnostic yield of 77%.
- Preventing unnecessary invasive procedures or receiving treatment without histological confirmation of cancer.
- Allows for treatment adaptation in two-third of the analyzed patient population.

INTRODUCTION

The number of solitary pulmonary nodules (SPNs) to be evaluated is expected to increase due to the introduction of lung cancer screening programs and the increasing amount of cardiac CT scans. Simultaneously it is necessary to improve diagnostic and therapeutic tools to approach the SPNs [1]. CT guided transthoracic procedures are the current gold standard for obtaining diagnostic biopsies of SPNs in the periphery of the lung [2]. Despite its accuracy in lesions of >20 mm, this technique is associated with a significant risk of complications [3,4]. Pneumothorax is reported in up to 26 % of cases, with need for chest tube insertion and hospitalization in up to 5.6 % of cases, and bleeding is reported in up to 18 % of cases [3–5]. The diagnostic yield of a CT guided transthoracic biopsy in selected peripheral lesions is around 75 % [6]. Alternative for a CT guided biopsy is Video- or Robotic-Assisted Thoracic Surgery with or without hookwire localization for wedge resection of SPNs located within 30 mm of the pleural surface [7]. Although a high diagnostic yield is reported, disadvantages are the invasiveness of the procedure and risk of conversion to a thoracotomy. Furthermore, this technique is not suited in case of a more centrally located SPN, as lobectomy is usually required.

Historically, lesions in the periphery of the lung are considered not accessible by conventional bronchoscopy [8]. To advance the range and diagnostic yield, and to improve safety of bronchoscopic procedures, several approaches have been developed using techniques like ultrathin bronchoscopy and radial endobronchial ultrasound (rEBUS) to confirm access to the SPN [9]. Guidance to the SPN was achieved with electromagnetic navigation bronchoscopy (EBN) and for verification of the correct position rEBUS, C-arm fluoroscopy or cone beam CT scanning were added [10–13]. Dependent on localization and size of the lesion, generally ENB reported a diagnostic yield of above 70 % and low complication rate with 2 % pneumothorax [10–13]. Additionally, in a substantial number of patients, clinicians still decide to irradiate a nodule or resect a lung lobe without histologic confirmation of an SPN in advance [14].

One of the newer techniques for obtaining diagnostic biopsies of SPNs uses virtual bronchoscopy navigation (VBN) to calculate the access to an SPN via a trans parenchymal route [15]. Here, the overall sensitivity to obtain a histopathologic diagnosis has been found to be around 77 % (72–82 %). The complication rate was low, with pneumothorax in 2 % of the cases and bleeding in 0.8 %, without additional safety issues in severe emphysema patients [11,16–19]. With this technique, in contrast to the CT guided transthoracic approach, also very small lesions (up to 7 mm diameter), and lesions that cannot be reached via the transthoracic route – located in the inner two thirds of the lung - can be approached. However, detailed clinical data, like the relation of the diagnostic

yield to the specific location of the pulmonary nodule, and data about the accessibility of nodules in a real-world clinical population are needed [1,20]. Because of the increasing number of nodules to be assessed, and to prevent patients from receiving treatment without histological confirmation of cancer [21], the aim of this study was to evaluate the performance of VBN to obtain a diagnosis of SPNs in a real-world clinical setting.

METHODS

We performed a single center, prospective, observational cohort study of patients undergoing the novel standard of care VBN procedure to assess an SPN – "The NAVIGATOR" – study. The protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen (UMCG) and registered centrally (UMCG METC 202100352, ClinicalTrials.gov identifier NCT05383105).

Patients with a suspicious pulmonary nodule were recruited in the Multidisciplinary Board of Thoracic Oncology of the UMCG and in the regional multidisciplinary boards. In these meetings potential procedures to obtain a sample of the SPN and technical aspects of these procedures were discussed. Patients were available for the VBN procedure when alternative procedures were considered not feasible based on expected yield, safety, and/or if previously performed procedures failed to establish a diagnosis. All patients provided informed consent for the procedure.

Additional inclusion criteria were: age > 18, pulmonary nodule(s) suspicious for malignancy or metastases of a known primary tumor, a distinct nodule with a diameter of > 6 mm in its largest dimension, nodule located in the parenchymal tissue > 5 mm from the parietal pleura and considered accessible by VBN.

Exclusion criteria were any contraindication to undergo bronchoscopy, inability to stop anticoagulants or antiplatelet medication around time of the procedure, pregnant or breastfeeding women, moderate to severe pulmonary fibrosis, severe emphysema with bullae > 5 cm in the vicinity of the target nodule or tunnel.

Before the procedure a dedicated high-resolution CT scan was performed from eligible subjects and assessed using the Archimedes VBN System (Broncus Medical, Inc., San Jose, California, USA) [22,23]. This image-guided navigation system comprises a workstation and software that reconstructs CT data into a 3D model, including the airways, blood vessels, ribs and lungs and provides features to mark the pulmonary nodule. The system calculates an airway path and suitable points of entry (POE) locations with a straight line,

vessel-free access to the pulmonary nodule (the tunnel path), as well as bronchoscopy paths for guiding the bronchoscopist to the POE locations [18,19,22,23].

During the procedure nodules were assessed with VBN in combination with fluoroscopy guidance and biopsies (preferred) or samples for cytology were obtained. Evaluation of a pneumothorax was performed with fluoroscopy at the end of the procedure. Specimen were evaluated by a dedicated pulmonary pathologist according to standard of care. The diagnostic yield was calculated according to the 'intermediate' definition by Vachani, et al, considering malignant and true benign outcomes as diagnostic and allowing for follow up on nodules [24]. After the procedure, results were discussed in the Multidisciplinary Board of Thoracic Oncology for each patient resulting in a definitive treatment proposal.

Patients characteristics including previously performed procedures and outcomes, as well as treatment plan without the VBN procedure, characteristics of the SPN, details of the procedure including but not limited to procedure time, radiation dose and duration of radiation, adverse events of special interest (respiratory failure, pneumothorax, subcutaneous emphysema, hemorrhage according to Common Terminology Criteria for Adverse Events (CTCAE v5) [25,26]), and treatment plan after the VBN procedure were recorded.

Given the nature of the study, descriptive statistics were applied using SPSSv23.

RESULTS

Between February 2021 and January 2022, 35 patients underwent the VBN procedure in our center. Patient and SPN characteristics are listed in Table 1. Main indications to request a biopsy were SPNs without a history of a solid malignancy (43 %), and SPNs in patients with a history of a solid malignancy other than lung cancer (37 %). In the minority of cases, a repeat biopsy was requested for mutation analysis in relapsing or progressive lung cancer harboring an oncogenic mutation. The majority of SPNs were solid lesions, mainly located in the upper lobes (66 %). About one third of the population underwent at least one diagnostic procedure before the VBN procedure.

Table 1. Patient and nodule characteristics NAVIGATOR

Total number of patients	**N = 35**
Age, median (range in years)	68 (45 – 80)
Gender, number (%)	
Male	18 (51)
Female	17 (49)
Indication for the procedure, number (%)	
SPN without history of solid malignancy	15 (43)
SPN in patients with history of solid malignancy other than lung cancer	13 (37)
Nodule, relapse/progression of prior lung cancer considered	7 (20)
Biopsy procedure before VBN procedure, number (%, multiple procedures per patient possible)	
None	22 (63)
Procedure before VBN	13 (37)
Diagnostic bronchoscopy	9
EBUS FNA	2
EUS FNA	1
CT guided transthoracic biopsy	3
Thoracoscopy	1
Morphology SPN, number (%)	
Solid	33 (94)
Spiculated	15
Lobulated	15
Cavitated	3
Subsolid	1 (3)
Ground glass opacity	1 (3)
Localization SPN, number (%)	
Right upper lobe	13 (37)
Middle lobe	2 (6)
Right lower lobe	8 (23)
Left upper lobe	10 (28)
Left lower lobe	2 (6)
SPN longest diameter, median (range; in mm)	24 (10 – 57)
SPN, grouped per diameter, number (%)	
Diameter ≤ 20 mm	12 (34)
Diameter > 20 mm	23 (66)
Bronchus sign visible, number (%)	22 (63)

SPN: solitary pulmonary nodule; VBN: virtual bronchoscopy navigation; EBUS: endobronchial ultrasound; FNA: fine needle aspiration; EUS: endoscopic ultrasound.

Table 2. Procedural characteristics NAVIGATOR

Procedure (bronchoscopy) time, median (range; in minutes)	43 (25 – 89)
Fluoroscopy time, median (range; in minutes)	2.5 (0.3 – 7.8)
Radiation dose during procedure, median (range; in mSv)	16.6 (0.7 – 85.5)
Chosen path to SPN, number (%)	
Airway path	18 (51)
Tunnel path	13 (37)
Both	4 (11)
Adverse events of special interest, number (% of procedures)	
Hemorrhage	9 (26)
Grade 1	5
Grade 2	2
Grade 3	2
Pneumothorax	-
Late subcutaneous emphysema*	1 (3)
Respiratory failure	-
Diagnostic yield of VBN procedure (%)	
Overall	77
Per SPN diameter	
Diameter ≤ 20 mm	37
Diameter > 20 mm	78
Per chosen path	
Airway path	89
Tunnel path	62
Both	75
Size of SPN grouped by VBN result, median (range; in mm)	
Diagnosis obtained	25 (10 - 57)
Diagnosis not obtained	18 (10 - 30)

SPN: solitary pulmonary nodule; VBN: virtual bronchoscopy navigation. *no intervention necessary

In Table 2 the procedural characteristics are given. The route with an airway path, tunnel path, or a combination, was chosen based on the navigational planning and at the discretion of the bronchoscopist. In half of the cases an airway path was chosen (51 %). Fig. 1 depicts a procedure with a tunnel path.

Adverse events of special interest were few and manageable (Table 2). Grade 3 hemorrhage according to CTCAE criteria, needing additional bronchoscopic hemostasis, occurred in two patients (6 %). One of these patients also needed noradrenalin due to hypotension with signs of secondary cardiac ischemia during the procedure. This patient was diagnosed with a primitive neuroectodermal tumor. In the second patient no diagnosis was obtained. Both patients recovered without any sequelae. In our case series no pneumothorax occurred, in one case however, three days after the VBN procedure a self-limiting subcutaneous emphysema of the neck region without other signs of a pneumothorax was diagnosed.

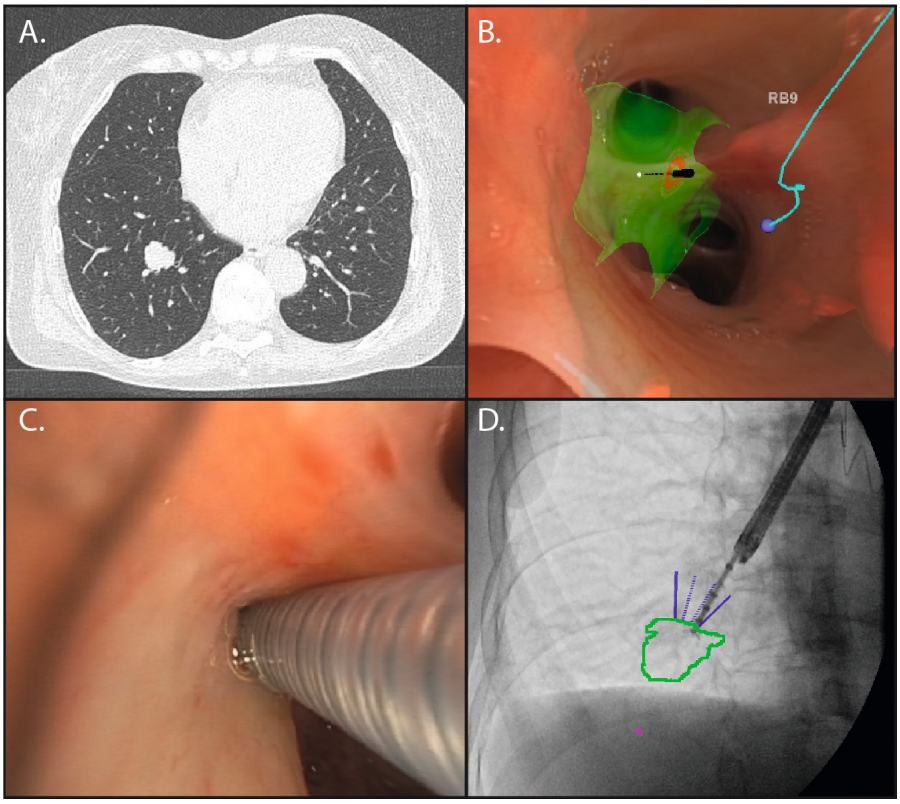

Figure 1. Procedure with a tunnel path. A. Solitary pulmonary nodule located in the right lower lobe (RB9), axial view on CT scan. B. Endoscopic view of RB9 with virtual overlays of airway path (blue), the intrapulmonary nodule (green) and the vessel-free location for puncture. C. Needle in subcarina of RB9 to access the intrapulmonary nodule. D. Fluoroscopy view of bronchoscope and tunnel sheet guided in direction to the nodule (green) with open forceps.

The overall diagnostic yield leading to a classifying diagnosis of the VBN procedure was 77 % (27/35 cases, Table 2). The diagnostic yield was dependent on SPN size and chosen path, with highest yield in lesions with an airway path on CT imaging 89 % (15/18 lesions), and 78 % in SPNs with a diameter > 20 mm (18/23 lesions). The median diameter of SPN with diagnosis was 25 mm (range 10–57).

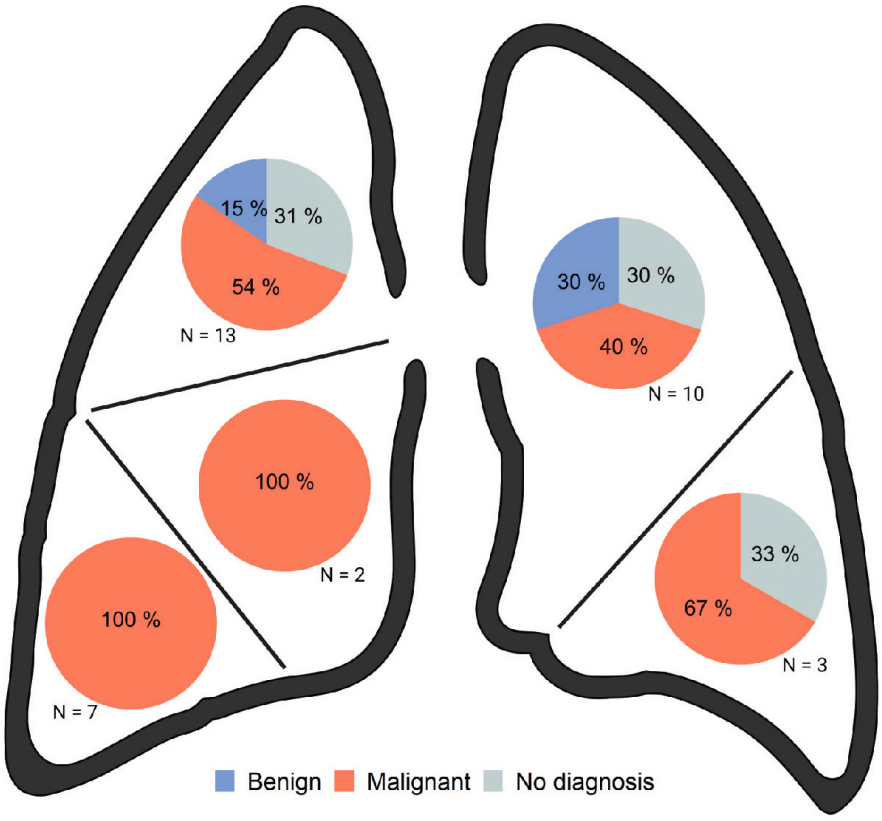

Figure 2. Distribution of diagnostic yield per lobe. N = number of procedures per lobe.

The diagnostic yield per lobe is reflected in Fig. 2. In 22 cases we established a malignancy, and in 5 cases a benign diagnosis. In all cases of malignancy, the obtained tissue was sufficient for additional molecular testing to aid treatment decisions. Two benign SPNs were based on an infection, one on a Streptococcus pneumoniae infection, and another on a Streptococcus mitis infection. One pulmonary nodule was formed by reactive changes of the lung tissue after chemotherapy, and was fully resolved in time. One lymphocytic SPN was considered malignant by the treating physician and the patient underwent stereotactic radiotherapy without a confirmative diagnosis of a malignancy. An SPN with eosinophilic inflammation was also considered malignant, and the patient went for thoracic surgery. In the resection specimen a typical carcinoid was found.

In all patients we proposed an a-priori advice for presumed treatment in case of no histological confirmation of the nodule (Table 3). After the VBN procedure, this treatment plan was adapted in 24 patients (69 %).

Table 3. Per case data NAVIGATOR

	Segment	Indication	Path	SPN largest diameter (mm)	Diagnostic procedures performed before VBN	Empiric treatment advise without VBN	Consequence of VBN (yes / no)	Definitive pathology diagnosis after VBN	Definitive treatment advise after VBN
1	RB3	SPNdd	AP	26	BS	Empiric RT in lung cancer dose (high dose)	yes	MALT-lymphoma	RT low dose, curative for lesion lymphoma
2	LB3	SPNpr NSCLC stage IVB, progression on EGFR-TKI	TP	41	BS	No certain options	yes	NSCLC, Adenocarcinoma EGFR-mutation exon 19 del, no resistance mechanisms.	Chemo-immunotherapy
3	LB6	SPNdd	TP	36	EBUS	TTP	yes	NSCLC, adenocarcinoma, EGFR mutation exon 19 del, PD-L1 = 80%. cT2aN3M0. No metastasis thyroid, no AML	Chemoradiotherapy
4	RB2	SPNpr NSCLC stage IVB, EGFR mutation exon 19 del. Progression on EGFR-TKI	TP	24	Thoracoscopy	TTP	no	Atypical cells	Chemo-immunotherapy because of NSCLC (by additional TTP: not sufficient tissue to reveal resistance mechanisms)
5	RB8	SPN	AP	57	None	EBUS or lobectomy	yes	NSCLC adenocarcinoma, no driver mutations. cT3N0M0	Lobectomy RLL + neo-adjuvant chemo-immunotherapy in study ypT1aN0PL1
6	RB1	SPNdd	AP	35	BS	Empiric SBRT	yes	NSCLC adenocarcinoma, cT2aN0M0, EGFR mutation exon 19 del, PD-L1 = 70%	High dose radiotherapy
7	RB9	SPN	TP	19	None	Empiric SBRT	yes	NSCLC adenocarcinoma, cT1bN0M0, no driver mutations, PDL1 = 0%,	Lobectomy RLL; adenocarcinoma, pT1bN0PL0R0

	Segment	Indication	Path	SPN largest diameter (mm)	Diagnostic procedures performed before VBN	Empiric treatment advise without VBN	Consequence of VBN (yes / no)	Definitive pathology diagnosis after VBN	Definitive treatment advise after VBN
8	RB1	SPNdd	AP+TP	26	None	Empiric SBRT	no	No diagnosis, nodule not reached.	Empiric SBRT in suspected lung malignancy cT1cN0M0 with partial response.
9	RB4	SPNpr NSCLC stage IVB with EGFR mutation exon 19 del. Progression on first generation EGFR-TKI	TP	20	None	None	yes	NSCLC adenocarcinoma, EGFR exon 19 del, EGFR T790M, no other resistance mechanisms.	Targeted treatment for EGFR T790M.
10	LB9	SPNdd	AP	22	None	High risk TTP	yes	Metastasis oropharynx carcinoma	Systemic therapy
11	LB1	SPNpr Suspected progression on chemoimmunotherapy in NSCLC IVB with EGFR exon 19 deletion	AP	22	None	High risk TTP	no	Reactive changes (was resolving nodule in follow up)	Empiric switch to afatinib due to progression in other lesions. Not histology proven.
12	LB1	SPNdd	AP	16	None	High risk TTP	yes	Focal pneumonia, Culture: infection with S. pneumoniae	Antibiotic therapy, resolved nodule.
13	RB9	SPNdd	AP+TP	25	None	High risk TTP	no	Metastasis of SCC, can either be lung or larynx.	BSC due to fast deterioration
14	RB5	SPNpr	TP	14	None	High risk TTP	yes	Dysplasia: atypical p40+ cells, suspected primary SCC of the lung.	Radical RT
15	RB3	SPN	AP	27	None	High risk TTP	yes	Lung SCC	Radical RT in study with immunotherapy
16	RB8	SPN	TP	35	TTP	Unclear	yes	Melanoma	Systemic therapy

Table 3. Continued

	Segment	Indication	Path	SPN largest diameter (mm)	Diagnostic procedures performed before VBN	Empiric treatment advise without VBN	Consequence of VBN (yes / no)	Definitive pathology diagnosis after VBN	Definitive treatment advise after VBN
17	LB6	SPN	TP	11	None	High risk TTP	no	Lung tissue, considered non-representative	Follow up: indolent (1 year follow up)
18	RB3	SPNdd	AP	11	None	High risk TTP	no	Bronchial epithelial tissue, cartilage and connective tissue.	Follow up: Considered metastasis of thyroid carcinoma.
19	LB1	SPN	AP	22	None	Resection of a brain metastasis	yes	NSCLC, adenocarcinoma, KRAS-mutation G13C, PD-L1 = 0% cT1cN0M1c	SBRT on brain metastases and lesion LUL + chemoimmunotherapy
20	LB 1	SPN	AP	25	EUS + TTP	Diagnostic resection	yes	NSCLC adenocarcinoma, cT1cN0M1b, EGFR mutation exon 21 insertion, PDL1 = 60%.	Targeted therapy in study
21	LB1	GGO	TP	25	BS	Diagnostic resection	yes	Lung tissue with epithelial tissue, oedema and chronic infiltration	Follow up
22	LB3	SPNpr	TP	19	None	Diagnostic resection or empiric SABR	no	Lung tissue, connective tissue and anthracosis.	Empiric SABR
23	RB3	SPN	TP	11	None	Follow up	yes	NSCLC, Adenocarcinoma, cT1bN0M0 with ALK-EML4 fusion, PD-L1 = 5%	Lobectomy RUL pT2N1PL0R0 + adjuvant chemotherapy
24	RB1	SPN	AP	20	BS	Empiric SBRT	yes	NSCLC Adenocarcinoma, cT1bN0M0, KRAS G12C mutation, PD-L1 = 0%.	SBRT
25	RB3	SPNdd	AP	30	None	Follow up	no	Lung tissue with fibrosis and macrophages.	Suspicion of NSCLC. Follow up

	Segment	Indication	Path	SPN largest diameter (mm)	Diagnostic procedures performed before VBN	Empiric treatment advise without VBN	Consequence of VBN (yes / no)	Definitive pathology diagnosis after VBN	Definitive treatment advise after VBN
26	RB6	SPN	TP	40	BS + EBUS	Resection	yes	NSCLC adenocarcinoma cT2aN2M0, no mutations, PD-L1 = 0%	Chemoradiotherapy
27	RB2	GGO with solid component	AP	40	BS + TTP	Diagnostic resection	yes	NSCLC adenocarcinoma cT2aN0M0, EGFR mutation exon 19 del, PDL1 = 0%	SBRT
28	LB1	SPNdd	TP	10	BS	Follow up	no	Reactive changes with fibrosis and bronchial mucosa	Follow up: indolent
29	LB3	SPNpr	AP	10	None	SBRT	yes	Focal pneumonia, Culture: infection with S. mitis	Follow up, resolving nodule
30	RB9	SPN	AP	48	None	BS	yes	NSCLC adenocarcinoma cT2bN0M1c, KRAS mutation G12C, PD-L1 = 0%	SBRT brain metastases + BSC (due to deterioration with COVID19 infection)
31	RB7	SPNdd	AP+TP	24	None	BS	yes	Lung SCC cT1cN0M0	High dose RT
32	RB2	SPNdd	AP	34	None	High risk TTP	yes	Primitive neuroectodermal tumor	High dose RT
33	RB2	SPN	AP	13	None	Resection	no	Eosinophilic pneumonia	Sublobar resection: typical carcinoid pT1bN0
34	LB1	SPNdd	AP	47	BS	Follow up	yes	Large cell neuroendocrine carcinoma cT3N2M0	Concomitant chemoradiotherapy

Table 3. Continued

	Segment	Indication	Path	SPN largest diameter (mm)	Diagnostic procedures performed before VBN	Empiric treatment advise without VBN	Consequence of VBN (yes / no)	Definitive pathology diagnosis after VBN	Definitive treatment advise after VBN
35	RB2	SPN	TP	17	None	Follow up	no	Non-malignant lymphoid lesion	SBRT (considered malignant)

M: male; F: female; RB: right bronchus; LB: left bronchus; AP: airway path; TP: tunnel path; EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitor; AML: acute myeloid leukemia; BS: bronchoscopy; TTP: transthoracic puncture; RT: radiotherapy; SBRT: stereotactic body radiotherapy; SABR: stereotactic ablative radiotherapy; NSCLC: non-small cell lung carcinoma; SCC: squamous cell carcinoma; PD-L1; programmed death ligand 1; PL: pleural invasion; R: resection; BSC: best supportive care; ECOG: Eastern Cooperative Oncology Group; PS: performance status; SPN: solitary pulmonary nodule; EUS: endoscopic ultrasound; EBUS: endobronchial ultrasound
SPN: solitary pulmonary nodule, usually suspicion of lung carcinoma.
SPNdd: SPN in the context of a differential diagnosis, such as MALT-lymphoma (#1), AML and history of thyroid carcinoma (#3 and 18), AML (#6), mouth carcinoma (#8), pharynx carcinoma (#10 and 13), breast carcinoma (#12), laryngeal carcinoma (#25), renal cell carcinoma (#28), neuroendocrine bladder carcinoma (#31), GIST (#32), melanoma (#34).
SPNpr: (suspected) relapse or progression of established lung carcinoma (NSCLC)
GGO: ground glass opacity
No TTP nor surgery due to localization, bleeding risk, borderline pulmonary function or technical restrictions.
Complications (in 7 out of 35 procedures) were usually manageable: bleeding grade 1 (#2, 3, 5 and 13), bleeding grade 3 (#25 and 32), self-limiting cutaneous emphysema (#11).

DISCUSSION

We investigated the value of the new VBN in our first series of 35 cases with a pulmonary nodule. In our study we only selected SPNs that were not otherwise accessible or for which other diagnostic procedures were considered less successful or less safe. With a diagnostic yield of 77 %, our findings are in line with previous data [22,27 –30]. The performance of our first cohort of VBN procedures was comparable to other studies, taking into account the differences in technique. Due to small patient numbers we need to extend our cohort to make data more robust. An important advantage of a successful VBN procedure is that patients obtain a definite tissue based diagnosis and therefore can be offered appropriate treatment, avoiding more invasive procedures or futile treatment. Without this VBN procedure almost all patients would not have had a definite diagnosis. In our set, the treatment plan of two third of the patients was adjusted based on the definitive diagnosis after the VBN procedure.

In this observational cohort study we confirmed that VBN can be performed with manageable adverse events. No pneumothorax or respiratory failures were observed. There was, however, one patient with subcutaneous emphysema 3 days after the procedure. Fluoroscopy after the procedure and a PET-CT scan one day after did not show any signs of a pneumothorax. In a multicenter study of 1388 patients in 37 centers, the VBN-related grade 2 or higher bronchopulmonary hemorrhage and grade 4 or higher respiratory failure rates were 1.5 % and 0.7 %, respectively [29]. In a single-center study assessing 114 nodules, pneumothorax occurred in 1.9 % and mild bleeding in 1.0 % [30]. In our study grade 2 and 3 bronchopulmonary hemorrhage rate was 11.5 %, with relevant (grade 3) hemorrhage occurring in two patients (5.7 %), which resolved without sequelae.

The diagnostic yield of bronchoscopic procedures is partly dependent on the presence of a bronchus sign [30–32]. A positive bronchus sign refers to the presence of a bronchus leading directly to a peripheral lung lesion, as observed on CT. A previous study using VBN reported an overall diagnostic yield of 67 % (34/51), increasing to 79 % (30/38) when only patients with a bronchus sign on CT were considered [31]. In cases without a bronchus sign, the reported yield was only 31 %. In our cohort, the diagnostic yield was also highest when considering only cases in which the SPN could be approached by an airway path (89 %). However, in contrast to earlier data, the diagnostic yield of procedures approaching lesions without a bronchus sign by following a trans parenchymal route, was greatly improved (62 %) [19,22]. In our study we created 17 tunnel paths between the central airways and the lesions. Thorough preparation, including a dedicated pre-procedural CT scan and constructing airway- and tunnel paths, was crucial to obtain a

diagnosis. Procedural issues possibly hampering the accurate planning of the virtual pathways to the nodule were resolution of the lesion on the pre-procedural CT scan, physical blockades like mucus impaction in smaller airways [33], mismatches occurring due to inadequate positioning of the patient on the table in comparison to the CT scan, as well as the difference between patient triggered deep inhalation during the scan and intraprocedural breath hold under anesthesia [34,35]. Especially in the lower lobes, the accordance of the appointed region of the nodule compared to the planning can be low. This discrepancy due to 'movement' of the pulmonary nodule during anesthesia is reported to be up to 2.5 cm when the nodule is located in the lower lobes [36]. Better imaging techniques such as cone-beam CT with body-shape sensing are available to overcome problems of respiration and CT-to-body divergence, and can increase diagnostic accuracy [13,37–39]. Additional confirmation of the position of the nodule can also be achieved with rEBUS which may contribute to an even higher diagnostic yield [40,41]. Additional localization confirmation is attributable for lesions in the right upper lobe, lesions not visible on fluoroscopy and lesions in the peripheral third of the lung [42–44]. Finally, improved localization of the nodule is also necessary to be able to safely apply local ablative therapies with minimal damage of healthy lung tissue in the future.

Next to the bronchus sign, size of the nodule is an important parameter in determining the diagnostic success of a procedure. In a large meta-analysis, a CT-guided biopsy was superior to VBN plus rEBUS for the evaluation of lesions smaller than 2 cm and located in the outer third of the lung [6]. For larger peripherally located lesions the endobronchial approach may be preferred, as it has a high diagnostic yield (80 %) and a low risk of procedure-related complications [6].

The location of the lesions in our cohort were not equally distributed over all lobes, with more lesions present in the upper lobes. This upper lobe predominance reflects the findings of screen-detected lung cancers in the NELSON trial, where 65 % of nodules were located in the upper lobes [20]. Furthermore, it indicates the difficulty to obtain a diagnosis via conventional bronchoscopy or CT-guided transthoracic biopsy in the apical segments of the upper lobes. Also procedures with VBN in the upper lobes are challenging due to angulation of the scope and related difficulties with advancing the forceps, brush or needle into the working channel. In our experience, use of ultrathin bronchoscopes can be disappointing due to little amount of tissue that can be obtained with the small biopsy tools. Endoscopic tools with greater flexibility, but large enough to obtain a sufficient amount of tissue, are still needed.

The additional value of the new VBN technique to be able to further personalize treatment of our patients, can only be achieved by an extra investment of time and

human resources. Pre-procedural CT scan and route planning, is followed by a procedure with median time of 50 min during which next to the anesthesiologist, a radiology technologist, the bronchoscopist, a 'co-pilot' for the navigation and endoscopy staff is needed [45].

CONCLUSIONS

In view of the expected lung cancer screening program leading to increasing numbers of especially small pulmonary nodules, better tools to reach SPNs are needed to help select the right treatment for the right patient. VBN with the possibility to also use a trans-parenchymal route is a new technique with a good diagnostic yield, also in patients in whom previously performed procedures failed to establish a diagnosis and/or alternative procedures are considered not feasible based on expected yield and/or safety. Preventing futile or more invasive procedures like surgery or transthoracic punctures with a higher complication rate is beneficial for patients. Using the new VBN technique, we reached a diagnostic yield of 77 %, and allowed treatment adaptation in two-third of the analyzed patient population.

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Chapter 10

Thesis in perspective and a glance at the future

The aim of this thesis was to investigate what role can be played by biomarkers in the treatment of lung cancer. What criteria do we test and what medication is available? Can biomarkers act as a target for precision therapy and can biomarkers help us evaluate therapy in order to detect early progression in a non-invasive manner? Will we be able to use biomarkers to stratify patients for specific treatment options?

NEUROENDOCRINE TUMORS AND NEUROENDOCRINE CARCINOMAS

In the first part of the thesis, we discussed the rare and difficult to treat neuroendocrine tumors (NETs) and neuroendocrine carcinomas (NECs). These syndromes have hardly benefited from all the new methods to better visualize cell biology and the unravelling of the genome. Medication is administered regardless of either tumor selection or patient selection. In this era of more and more treatment options becoming available, such as immune checkpoint inhibition, it is necessary to improve the methods for selecting the best therapy available for lung cancer patients. Repurposing of medication, the use of medication that is registered for another indication, is another option that needs to be investigated. Conducting studies of tumor selection or patient selection based on biomarkers is the way to proceed. After all, in the absence of patient or biomarker selections, agents that fail once might nevertheless have a beneficial effect in another group of patients.

In **chapter 2,** we reviewed the recent progress achieved in the field of staging and treatment of small cell lung cancer (SCLC) with surgery and radiotherapy. Furthermore, we reported on advances made in systemic treatment such as immunotherapy and its role in the treatment of SCLC in the last decade. We provided an overview of the treatment options and future perspectives in the era of molecular analysis on dividing SCLC into four molecular subtypes associated with therapeutic sensitivities.

In **chapter 3** we wrote an editorial for a special issue on “Targeted therapy for small cell lung cancer”, aiming to reveal targetable biomarkers, as well as biomarkers that can stratify patient groups for more effective treatments. We described three main directions moving forward to better stratify patients for specific therapies and to overcome tumor heterogeneity. The classification of SCLC into four molecular subgroups was the first step. The second solution may be in applying combination therapy rather than monotherapy. Thirdly, novel drug delivery systems should help to target tumor cells whilst sparing healthy cells.

Combination of treatment modalities to overcome tumor heterogeneity in SCLC, classification of SCLC into 4 molecular subtypes with their own therapeutic vulnerabilities, and development of novel drug delivery systems is the way forward to the treatment of this devastating and prevalent disease

Genetic testing of SCLC

Recently, genetic testing in the largest real-world cohort of 3,600 SCLC patients identified new genetic alterations and subtypes, making a case in favor of personalized treatment in the future [1]. Unique in this study was the combining of genetic data with clinical data, such as overall survival. This allowed for revealing genomics by metastatic site. Tumor mutational burden (TMB) and *PTEN* mutations were more prevalent in brain metastases compared to primary tumors and liver metastases. In a cohort of patients harboring an Serine/threonine kinase 11 (*STK11*) mutation, an association with a worse overall survival was found. Patients with *STK11* mutations may benefit from efforts made in other cancer types to develop new therapeutic approaches for this mutation. Furthermore, a cohort of *TP53*/*RB1* wild type was defined, about 5% of all SCLC. In some of these, human papilloma virus (HPV) was prevalent. Transformed SCLC with driver mutations typically found in NSCLC emphasizes the need for re-biopsy in case of progression to treatment. This study focused on the need for unravelling the biology and on the search for markers in order to enable tailored treatment. Overlaps in biomarkers or genes found in other tumor types warrant expansion of therapies to these other tumor types, for example, the development of STK11 inhibitors to treat SCLC and NSCLC harboring an *STK11* mutation.

In **chapter 4** we conducted a systematic review of O6-Methylguanine-DNA methyltransferase (MGMT) in lung cancer to evaluate whether MGMT promoter methylation can act as a prognostic or predictive biomarker to select patients with lung cancer who can benefit from treatment with temozolomide. The conclusion of the review is that MGMT promoter methylation in NSCLC is not a prognostic, nor a predictive factor, hence temozolomide has no place. In SCLC and NET patients with MGMT promoter methylation, it still needs to be confirmed whether it can serve as a predictive biomarker for treatment with temozolomide. In these cases, temozolomide can be considered as a “personalized” therapy.

In **chapter 5**, we performed a retrospective analysis to establish the frequency of MGMT promoter methylation and ALK expression in tissue samples of 75 patients with NETs and NECs. Ten of 70 (14%) specimens were ALK IHC positive. The ten ALK IHC positive specimens consisted of two typical carcinoids, two atypical carcinoids, and six SCLC. None of the 13 LCNECs were ALK IHC positive. ALK IHC positive specimens were tested for ALK

FISH. None of them showed rearrangements. In 5 tissues of high ALK expression, the presence of *ALK* mutations was tested, but no *ALK* mutations were identified. We found that MGMT promoter methylation was present in 33% (3/9) of patients with typical carcinoid, in 22% (2/9) of patients with atypical carcinoid, in 22% (8/37) of patients with SCLC and in 8% (1/12) of patients with LCNEC. We concluded that routine testing of NET and NEC samples for an ALK rearrangement is not recommended as ALK expression is not associated with an ALK rearrangement. Routine testing of NET and NEC samples for MGMT will detect a promoter hypermethylation in a sizable minority of patients who are eligible for targeted treatment with temozolomide.

ALK expression in NET and NEC reflects the origin of the tumor, the neuroendocrine crest, and has no clinical consequences, so should not be tested in NET and NEC

MGMT can act as a biomarker to predict response to temozolomide in NET and NEC

MGMT promoter methylation

In general, MGMT hypermethylation and NSCLC were found to be associated, but this had no impact on overall survival in NSCLC patients [2]. In an observational retrospective study, the treatment of neuroendocrine neoplasms with temozolomide resulted in an overall response rate of 27.4% [3]. Correlation was found with MGMT promoter methylation status, overall response rate being 51.8% and without MGMT promoter methylation 17.7%. This finding was prospectively confirmed in patients with neuroendocrine neoplasms, of which 23% lung NETs and 64% pancreas NETs [4]. Patients in the methylated group had longer PFS (median not reached) versus 30.2 months in the unmethylated group. The overall response rate was 60% in the methylated group and 24% in the unmethylated group. This was not statistically significant, due to the small patient group. Another multicenter study randomized 111 patients with advanced duodeno-pancreatic, lung, or NETs of unknown primary site for treatment with an alkylating agent or oxaliplatin-based therapy, stratifying patients according to MGMT-methylation [5]. The study is closed and results are awaited. In pancreatic NETs, treatment with temozolomide plus capecitabine revealed benefits exceeding temozolomide alone [6]. MGMT deficiency was associated with response to treatment. The authors conclude that routine testing of MGMT is not recommended, but can be considered to select patients for treatment with capecitabin and temozolomide.

Repurposing of drugs

In rare tumors with high medical need, it is important to overcome cancer-specific properties and to aim for targets that can influence the outcome of therapy [7]. Emerging targets like DLL3 in SCLC are also detected in NETs and LCNEC [8, 9]. These targets are not only important as a guide for treatment options [10], but also expand the range of therapeutic possibilities for LCNEC and NETs [11]. A study with ADC Rova-T revealed an ORR of 13% in DLL3-expressing NECs and NETs [12].

Studies with new medication or new targets for SCLC, such as lurbinectedin plus the ATR kinase inhibitor berzosertib, the antibody drug conjugates (ADCs) ABBV-706 and ABBV-011, multiple BiTes and CAR-T, are now including patients with NETs and LCNEC, and will thereby hopefully enlarge the therapeutic arsenal in all NETs [13-19].

K-RAS MUTATED NSCLC

In the second part of the thesis, we conducted feasibility studies in a cohort of patients with advanced Kirsten rat sarcoma viral oncogene homologue (*K-ras*) mutated NSCLC. In the early days of treatment with immunotherapy in NSCLC, PD-L1 was used as a biomarker to predict responses to therapy [20]. However, when we started the study in 2015, we did not use PD-L1 to make therapy decisions, *K-ras* mutation was mandated to enter the study subject to fulfilment of the other inclusion criteria.

In **chapter 6,** we described the trial in which we aimed to assess the clinical relevance of monitoring ctDNA in blood samples of patients with *K-ras* mutated advanced NSCLC, who were treated with immunotherapy in order to detect early responses and to enable the prediction of long-lasting responses. Altogether, decreasing mutant copies estimated with droplet digital PCR were associated with longer PFS and OS compared to patients displaying increased or stable ctDNA levels. CtDNA dynamics in combination with PD-L1 status is a promising cost-effective approach to monitor durable clinical benefit, PFS and OS in patients with advanced NSCLC treated with immunotherapy. Measuring a single tumor-derived molecular aberration, when retrieved from the circulation, improves the early recognition of a durable clinical benefit, and can assist in treatment decision making.

Monitoring ctDNA dynamics is an easy-to-use and promising monitoring tool

In **chapter 7,** we investigated the gut microbiome in a Dutch cohort of 33 patients treated with anti-PD-1 immunotherapy for advanced *K-ras*-mutated NSCLC. We presented taxa and pathways associated with response to immune checkpoint inhibition and immune-related adverse events in this homogeneous cohort of patients. We found an overlap

in microbial signatures of response and treatment resistance in a cohort of melanoma patients, suggesting shared signals across different tumor types. The gut microbiome potentially reflects general mechanisms and microbiome signatures and is therefore tumor-independent.

The gut microbiome with its specific taxa and pathways potentially reflects general mechanisms and microbiome signatures, and is therefore tumor independent

Detection of *K-ras* mutations

Tumor tissue is the "gold standard" to detect *K-ras* mutations. However, tissue is not always available, whilst drawing a blood sample from the patient is less invasive. Identification of *K-ras* G12C mutations in circulating tumor DNA was feasible and the concordance between ctDNA and tumor tissue was high, around 98% [20, 21]. The most common co-occurring mutation found was *STK11.* Detection of *K-ras* mutations in plasma can act as a surrogate for tissue samples, although these should be measured regularly and test results should be available within a reasonable timeframe to make clinical decisions for the patient [21, 22].

Nowadays, using whole exome sequencing to examine NSCLC, we found that more factors are associated with responses, such as tumor mutational burden (TMB) [23]. Mutations such as *STK11*/ liver kinase B1 (*LKB1*) and Kelch-like ECH-associated protein 1 (*KEAP1*) are not associated with any durable clinical benefit of immunotherapy [24, 25]. However its impact on PD-(L)1 inhibition in *K-ras*-mutated advanced NSCLC patients is unknown [26]. All these mutations and genomic alterations need to be further investigated in prospective studies.

Targeting *K-ras* mutations

K-ras mutation used to be hard to target in the past decades [27]. Recently, a lot of attention has been drawn to the *K-ras* G12C mutation, since effective inhibition of *K-ras* G12C is possible with sotorasib and adagrasib [28, 29]. Sotorasib produced an ORR of 37% to 43% in pretreated advanced NSCLC with *K-ras* G12C mutation [28]. Furthermore, sotorasib was better than docetaxel in phase 3 with improved PFS and ORR [30]. Although clinical outcomes vary from early disease progression in 5 to 16% of patients to durable responses with an 2-year OS rate of 32.5% [31].

The largest cohort of NSCLC patients with a *K-ras* G12C mutation consisted of 424 patients treated with sotorasib or adagrasib [32]. Co-mutations seen in the patient group with early progression (< 3 months) were *KEAP1*, *SMARCA4*, *CDKNA2A*, and *STK11*. *TP53*

was the most frequently co-mutated gene in the cohort, but was not associated with clinical outcomes. DNA damage repair (DDR) genes such as we saw in SCLC: *ATM*, *ATR* and *CHK1*, provided a higher ORR (52.2% vs 27.7%) and a significantly longer PFS (5.9 months vs 4.6 months) with *K-ras* G12C compared to DRR wild type. Only numerical OS was found, though not in a statistically significant degree.

Immunotherapy was less effective in *STK11* and *KEAP1* mutant lung cancer [26]. STK11 loss resulted in the silencing of STING in KRAS, which downregulates immunogenicity in *K-ras* mutated NSCLC [33]. Furthermore, *STK11* mutation was associated with a lack of PD-L1 expression, reduced tumor-infiltrating cytotoxic CD8+ T lymphocytes and resistance to immune checkpoint inhibition in patients harboring a *K-ras* mutation [24, 34]. *KEAP1* seemed a negative predictive biomarker for the response to immunotherapy, although correlated with high tumor mutational burden (TMB) levels, association was also found with lower immune infiltrates suggestive of a cold tumor immune microenvironment [35].

In a cohort of 1,261 patients with NSCLC, *K-ras* mutation was detected in 536 patients (42.5%), *STK11* mutation was detected in 20.6% and *KEAP1* mutation in 19.2% of cases [36]. Co-occurring mutations were found in around 10%. In the KRAS group, harboring *STK11* and *KEAP1* mutations had significantly lower PD-L1 expression. TMB was higher in de *STK11* and *KEAP1* group. *STK11* and *KEAP1* are also predictive of worse outcomes when treated with chemotherapy. However, in this study, *KEAP1* co-occurring with *K-ras* G12C is associated with poor prognosis and inferior outcome. Co-mutation of *STK11* in the absence of *KEAP1* did not affect the ORR, PFS, or OS with *K-ras* G12C. A study among 330 patients found *TP53* (42%), *STK11* (29%) and *KEAP1* (27%) [36]. A significantly shorter survival was seen in co-mutation *KEAP1*, both in treatment with chemotherapy and in immunotherapy. The co-mutation status of *STK11* and *TP53* was not associated with survival.

Whether the presence of *STK11* and *KEAP1* mutations predicts a worse outcomes on immunotherapy alone or in combination with other treatments, such as targeted therapy, chemotherapy or radiotherapy, will have to be evaluated in a prospective manner. At this point, they are prognostic rather than predictive.

However, other *K-ras* mutations exhibit different clinical outcomes, suggesting a different underlying biology [37]. For example, *K-ras* G12D mutations were prevalent in never smokers compared to *K-ras* G12C. In this group, treatment with immunotherapy alone was associated with a worse outcome, ORR 15.8% vs 28.4% in the *K-ras* mutated group overall. Treatment with chemoimmunotherapy was superior in this group. New clinical

trials will have to lead to stratification of different *K-ras* mutations or co-occurring mutations.

With the studies covered by this thesis, we have been able to gain a more profound insight into novel biomarkers in lung tumors. Biomarkers are of vital importance to select and stratify patients for specific treatments.

TISSUE

Meanwhile, obtaining tissue remains of the utmost importance. Not only to diagnose a pulmonary nodule, but also to make a choice between the new treatment options that have become available lately. Diagnostic techniques for assessing pulmonary lesions remain challenging. Newer bronchoscopy techniques, such as virtual bronchoscopy navigation, to obtain tissue from difficult-to-reach abnormalities in the lung, it is now possible to carry out complete molecular analysis and to offer appropriate treatment. Or, in the case of progressive disease of the growing abnormality, these techniques allow us to obtain new tissue and assess resistance mechanisms for a different choice of therapy. Sufficient tissue is important for molecular analysis in the development towards precision medicine.

In **chapter 8** we presented a 'Lesson of the Month' about an elderly patient with chronic myelomonocytic leukemia, who was diagnosed with lung adenocarcinoma. In the lung biopsy, several mutations were revealed, a *PIK3CA* mutation, an uncharacterized *MET* frameshift mutation and an *IDH2* mutation. The latter originated from tumor-infiltrating chronic myelomonocytic leukemia. The two other mutations derived from the lung cancer. The *PIK3CA* mutation is a well-known driver mutation in lung cancer, the *MET* frameshift mutation was shown to induce *MET* exon 14 skipping, which was successfully targeted with crizotinib. This case illustrates that seemingly unexpected mutations can derive from an infiltrating second malignancy, which might not be recognized by histomorphology alone. The presence of a second malignancy should be considered when an unexpected genetic variant is detected in the molecular analysis of solid malignancies.

In **chapter 9,** we conducted a single-center, prospective, observational study – NAVIGATOR - of patients undergoing a virtual bronchoscopy navigation (VBN) procedure to assess a pulmonary nodule. We reported on a cohort of 35 consecutive patients who entered our program. The nodules were "hard to get", considered not reachable by a CT-guided transthoracic biopsy or conventional bronchoscopy or with EBUS. The overall diagnostic yield was 77% and was dependent on the size of the nodule and chosen path.

Adverse events were few and manageable. Preventing more invasive procedures such as surgery or transthoracic punctures with a higher complication rate is beneficial for patients. Base on performing VBN with a diagnostic yield of 77%, we allowed a treatment adjustment in two-thirds of the analyzed patient population.

Novel approaches to assess tumor tissue in (suspected) lung cancer patients are feasible

In the rapidly evolving field of interventional bronchoscopy, we concluded in our NAVIGATOR study that we still need to improve the diagnostic accuracy and additional localization confirmation of the position of the nodule. Addition of radial-EBUS and cone-beam CT-scan during the procedure is strongly recommended [38]. Not only in order to avoid complications, such as bleeding and pneumothorax, but, more importantly, to increase the diagnostic yield of the procedure. To build a solid database, efforts are being undertaken to expand to multicenter logistics with other expert centers.
Secondly, the correlation with detailed clinical data is expected to help selecting patients for this procedure. Furthermore, better selection criteria are required to allocate patients for the diagnostic tool that is most appropriate to obtain tissue, namely the NAVIGATOR or a CT-guided transthoracic biopsy. Comparison of data of extended numbers of procedures in NAVIGATOR with existing data of patients undergoing CT-guided transthoracic punctures at our center in the last 5 years can facilitate the development of selection criteria to allocate patients for the safest procedure with a priori the highest diagnostic yield.

Virtual bronchoscopy navigation

Virtual bronchoscopy has advantages in providing information of the airways, vessels and pleura. The planning CT is of the utmost importance since the virtual environment relies on it. The procedural issues that possibly hampered the accurate planning of the virtual pathways to the nodule were resolution of the lesion on the pre-procedural CT scan, and physical blockades like mucus impaction in smaller airways [39]. Moreover, CT to body divergence is a major obstacle to successful navigation [39]. This means that pre-procedural lung volumes on the planning CT can differ drastically from intraprocedural lung volumes under general anesthesia in the intubated patient [40]. Especially in the lower lobes, the conformity with the appointed region of the nodule compared to the planning can be low. Nodules can move in position by 2 cm [41], mismatches can occur due to inadequate positioning of the patient on the table in comparison with the CT scan, and there can be a difference between patient-triggered deep inhalation during the scan and intraprocedural breath held under anesthesia [40]. More effective imaging techniques, such as the cone-beam CT with body-shape sensing, are available to bring

problems of respiration and CT-to-body divergence under control and can improve diagnostic accuracy [42].

Robotic-assisted bronchoscopy

To overcome limitations in navigation and diagnostic sampling, several robotic-assisted bronchoscopy (RAB) platforms were developed. Two platforms to perform RAB navigation received FDA approval and are currently available in the USA [43]. In Europe, no clearance for patient care has been received, as a result of which platforms are only available for research purposes at this moment. The first, the Monarch RAB platform (Auris Health, Inc., Redwood City, CA, USA) is based on electromagnetic navigation technology (EMN) [44]. The Ion Robotic-Assisted Endoluminal Platform (Intuitive Surgical, Inc., Sunnyvale, CA, USA) is based on shape-sensing technology, using fiber-optic bend sensors within the catheter itself to maintain orientation [45]. A third platform, the Galaxy System™ (Noah Medical, San Carlos, CA, USA), which is expected to obtain FDA clearance in 2023, provides EMN and digital tomosynthesis guidance on top of a disposable bronchoscope [43].

The main advantage of RAB navigation is the maintenance of a static position to navigate into very small peripheral airways under continuous visualization [46]. Navigational success was 88.6% with a diagnostic yield of 69.1% in the first study of EMN RAB [47]. Safe catheter positioning in the proximity of the lesion was feasible and safe (in Ion) [48]. Proximity of the catheter to the target lesion was the strongest predictor for a diagnosis, independent of the presence of a bronchus sign, or a concentric radial EBUS view [49]. Addition of the cone-beam CT for confirmation of the correct navigation and tool-in-lesion during biopsy sampling improved the diagnostic yield to 83% [50]. This emphasizes the issue of whether diagnostic yield is the correct primary endpoint, and that diagnostic accuracy is a better outcome measure, allowing for follow-up [51]. Overall, RAB navigation to the lesion with a bronchus sign, confirmation of the right location of the biopsy tool and obtaining sufficient tissue for diagnosis are all feasible [52]. Stability and precision are the keywords to reach peripheral lesions that are otherwise not capable of being diagnosed when using a robotic-assisted bronchoscopy platform.

In RAB platforms, CT to body divergence remains a major obstacle to successful navigation [42]. Augmented imaging, such as fluoroscopy or cone-beam CT, may resolve this problem. To improve the localization check, the O-arm CT was added [53]. With a view to overcoming these disadvantages, the Ion platform with shape-sensing ability turns out to be the most practical at this moment.

The drawback of robotic systems is that they are expensive, whilst publications on initial cost, maintenance and procedural tools are not available. Prospective head-to-head comparative studies of RAB and EMN have not yet been performed, and, retrospectively, the diagnostic yield is about comparable [54]. Information about cost development and the insurance-reimbursement options for robotic systems needs to be obtained before their introduction in daily patient care. On the other hand, it is beyond doubt that lung nodules will need to be reached and diagnosed with more precision in the future.

Future in bronchoscopy

As time goes by, the navigational bronchoscopy procedures will offer access to novel advanced imaging techniques, such as confocal laser endomicroscopy and fluorescence molecular endoscopy, which will facilitate the identification of the lesion during the endoscopic procedure [55]. Both techniques could be combined with laser-enhanced fluorescent-labelled tracer detection to assess sensitivity to medication [56, 57]. Finally, improved localization of the nodule will also be necessary in the future to be able to safely apply local ablative therapies with minimal damage to healthy lung tissue. Treatment of the malignant nodule by means of microwave ablation under the guidance of a navigation bronchoscopy or RAB guided biopsy will be the subject of future studies [58, 59].

Ultimately, we expect that the development and combination of these techniques will pave the way to a one-stop-shop approach, with rapid on-site evaluation of the biopsy and imaging, followed by local treatment of the malignant pulmonary nodule.

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Hoofdstuk 11

Proefschrift in perspectief en een blik op de toekomst

Het doel van dit proefschrift was te onderzoeken welke rol biomarkers kunnen spelen bij de diagnostiek en behandeling van longkanker. Op basis van welke criteria testen we en welke medicijnen zijn beschikbaar? Kunnen biomarkers bij longkanker fungeren als doelwit voor precisietherapie en kunnen biomarkers ons helpen de therapie te evalueren om vroegtijdige progressie van hun ziekte op minimaal invasieve manier op te sporen? Zullen we biomarkers kunnen gebruiken om patiënten de juiste behandeling te kunnen aanbieden?

NEUROENDOCRIENE TUMOREN EN NEUROENDOCRIENE CARCINOMEN

Het eerste deel van het proefschrift gaat over de zeldzame en moeilijk te behandelen neuro-endocriene tumoren (NETs) en neuro-endocriene carcinomen (NECs). Deze ziektebeelden hebben nog nauwelijks geprofiteerd van alle nieuwe methoden om de celbiologie en de ontrafeling van het genoom beter in kaart te brengen. Medicatie wordt toegediend ongeacht selectie op basis van tumorbiologie of patiëntkarakteristieken. In dit tijdperk waarin steeds meer behandelopties beschikbaar komen, zoals immuuntherapie, is het noodzakelijk de methoden te verbeteren die helpen selecteren om patiënten optimale therapie voor te schrijven. Het toedienen van medicatie die voor een andere indicatie werd geregistreerd, is een andere mogelijkheid die onderzocht moet worden. Het uitvoeren van onderzoek op basis van tumorselectie of patiëntselectie gebaseerd op biomarkers is dan een logische keuze. Immers, medicatie die heeft gefaald in een bepaalde patiëntengroep kan toch een gunstig effect hebben indien een andere selectie wordt toegepast, bijvoorbeeld op basis van biomarkers of andere specifieke patiëntkenmerken.

In **hoofdstuk 2** beschreven we de recente vooruitgang die is bereikt op het gebied van stadiëring en behandeling van kleincellig longcarcinoom (SCLC) met chirurgie en radiotherapie. Bovendien rapporteerden we over de vooruitgang die de afgelopen tien jaar is geboekt op het gebied van systeembehandelingen, zoals immuuntherapie en de rol ervan bij de behandeling van SCLC. We gaven een overzicht van de behandelopties en toekomstperspectieven in het tijdperk van moleculaire analyse. De indeling van SCLC in vier moleculaire subtypen die verband houden met therapeutische gevoeligheid is een aanknopingspunt voor verder onderzoek.

In **hoofdstuk 3** schreven we een introductieartikel (editorial) voor de speciale uitgave *"Targeted therapy for small cell lung cancer"* van het tijdschrift *Cancers*. In deze speciale uitgave verschenen artikelen waarin de auteurs op zoek gingen naar biomarkers die als doel kunnen dienen voor precisietherapie en naar biomarkers die patiëntengroepen

kunnen selecteren voor effectievere behandelingen. In dit artikel beschreven we drie belangrijke richtingen om patiënten beter te stratificeren voor specifieke therapieën en om de heterogeniteit van tumoren te overwinnen. De classificatie van SCLC in vier moleculaire subgroepen was de eerste stap. De tweede benadering kan liggen in het toepassen van combinatietherapie in plaats van monotherapie. Ten derde zouden nieuwe methoden voor medicijnafgifte in de tumorcellen of in de nabijheid van de tumorcellen moeten helpen om deze doelgericht aan te pakken en tegelijkertijd gezonde cellen te sparen.

Combinatie van behandelmodaliteiten om de heterogeniteit van tumoren in kleincellig longcarcinoom (SCLC) te overwinnen, classificatie van SCLC in 4 moleculaire subtypen met hun eigen therapeutische gevoeligheid, en de ontwikkeling van nieuwe methoden voor medicijnafgifte is de weg voorwaarts in de behandeling van deze verwoestende ziekte.

Genetisch onderzoek in SCLC

Onlangs werden in het grootste cohort van 3600 SCLC-patiënten nieuwe genetische veranderingen en subtypes geïdentificeerd, wat pleit voor gepersonaliseerde behandeling in de toekomst [1]. Deze studie richtte zich allereerst op de noodzaak de biologie te ontrafelen en vervolgens op de zoektocht naar markers om behandeling op maat mogelijk te maken. Uniek in dit onderzoek was de combinatie van genetische gegevens met klinische gegevens, zoals de algehele overleving. Daarnaast werd het mogelijk om van elke metastase het genoom te onthullen. Tumor mutational burden (*TMB*) en *PTEN*-mutaties kwamen vaker voor in hersenmetastasen vergeleken met primaire tumoren en levermetastasen. In een cohort patiënten met een serine/threoninekinase 11 (*STK11*) mutatie werd een verband met een slechtere algehele overleving gevonden. Patiënten met *STK11*-mutaties kunnen profiteren van inspanningen die bij andere soorten kanker worden verricht om nieuwe medicatie voor deze mutatie te ontwikkelen. Het aantreffen van biomarkers of genen die gevonden worden in andere tumortypen rechtvaardigen uitbreiding van therapieën naar deze andere tumortypen, bijvoorbeeld de ontwikkeling van STK11-remmers voor de behandeling van SCLC en niet-kleincellig longcarcinoom (NSCLC) die een *STK11*-mutatie herbergen.

Hoofdstuk 4 is een systematische review ter evaluatie van de aanwezigheid van O^6-Methylguanine-DNA-methyltransferase (MGMT) bij longkanker. De tweede vraag was of MGMT-promoter methylatie kan fungeren als een prognostische of predictieve biomarker om patiënten met longkanker te selecteren die baat kunnen hebben bij behandeling met temozolomide. De conclusie van het hoofdstuk is dat MGMT-promoter methylatie bij NSCLC geen prognostische of predictieve factor is, en dat voor temozolomide geen plaats

is in de behandeling. Bij SCLC- en NET-patiënten met MGMT-promoter methylatie moet nog worden bevestigd of dit kan dienen als voorspellende biomarker voor behandeling met temozolomide. In deze gevallen kan temozolomide worden beschouwd als een "gepersonaliseerde" therapie.

In **hoofdstuk 5** voerden we een retrospectieve analyse uit om de frequentie van MGMT-promoter methylatie en ALK-expressie te bepalen in weefselmonsters van 75 patiënten met NETs en NECs.

Tien van de 70 (14%) monsters toonden ALK-expressie met behulp van immuunhistochemie (IHC). De tien ALK-positieve monsters waren verdeeld in twee monsters met typisch carcinoïd, twee met atypisch carcinoïd en zes met SCLC. Geen van de 13 grootcellig neuroendocriene carcinomen (LCNECs) was ALK-positief. ALK IHC-positieve exemplaren werden getest met ALK FISH voor het aantonen van een ALK-fusie. Geen van hen vertoonde een ALK-fusie. In 5 weefsels met hoge ALK-expressie werd op de aanwezigheid van *ALK*-mutaties getest, maar deze werden niet gevonden. We concludeerden dat het routinematig testen van NET- en NEC-monsters op een ALK-fusie niet wordt aanbevolen, omdat ALK-expressie niet geassocieerd is met een ALK-fusie.

We ontdekten dat MGMT-promoter methylatie aanwezig was bij 33% van de patiënten met typisch carcinoïd, bij 22% van de patiënten met atypisch carcinoïd, bij 22% van de patiënten met SCLC en bij 8% van de patiënten met LCNEC. Routinematig testen van NET- en NEC-monsters op MGMT zal een promotor hypermethylatie detecteren bij een aanzienlijke minderheid van patiënten die in aanmerking zou kunnen komen voor een gerichte behandeling met temozolomide.

ALK-expressie in NET en NEC weerspiegelt de oorsprong van de tumor, de neurale lijst, en heeft geen klinische consequenties, dus hoeft niet te worden getest in NET en NEC.

MGMT kan fungeren als een biomarker om de respons op temozolomide in NET en NEC te voorspellen.

MGMT promoter methylatie

In het algemeen blijken MGMT-hypermethylatie en NSCLC geassocieerd te zijn, maar dit heeft geen relatie met de algehele overleving bij NSCLC-patiënten [2]. In een observationeel retrospectief onderzoek resulteerde de behandeling van neuro-endocriene nieuwvormingen met temozolomide in een totaal responspercentage van 27,4% [3]. De methylatiestatus van de MGMT-promoter bleek van belang, het totale responspercentage was hoger met MGMT-promoter methylatie (51,8%) dan zonder

methylatie (17,7%). Deze bevinding werd prospectief bevestigd bij patiënten met NETs, waarvan 23% long-NETs en 64% pancreas-NETs [4]. Patiënten in de gemethyleerde groep hadden een langere progressievrije overleving waarbij de mediaan nog niet werd bereikt in vergelijking met 30,2 maanden in de niet-gemethyleerde groep. Het totale responspercentage was 60% in de gemethyleerde groep en 24% in de niet-gemethyleerde groep. Dit was niet statistisch significant vanwege de kleine patiëntengroep. Een ander multicentrum onderzoek randomiseerde 111 patiënten met gevorderde duodeno-pancreas-, long- of NETs met onbekende primaire lokalisatie voor behandeling met een therapie gebaseerd op alkylering of op oxaliplatin, waarbij de patiënten werden gestratificeerd op basis van MGMT-methylatie [5]. Het onderzoek is gesloten en de resultaten worden afgewacht. Bij pancreas-NETs liet behandeling met temozolomide plus capecitabine voordelen zien ten opzichte van behandeling met alleen temozolomide [6]. MGMT-deficiëntie was geassocieerd met respons op de behandeling. De auteurs concluderen dat routinematig testen van MGMT niet wordt aanbevolen, maar wel kan worden overwogen patiënten hierop te selecteren voor behandeling met capecitabine en temozolomide.

Veranderen van indicatie voor toediening van medicijnen

Bij zeldzame tumoren is het belangrijk kankerspecifieke eigenschappen te overwinnen en te richten op biomarkers die de uitkomst van de therapie kunnen beïnvloeden [7]. Een in opkomst zijnde biomarker zoals delta-like ligand 3 (DLL3) in SCLC wordt ook gevonden in NETs en LCNEC [8, 9]. Deze biomarkers zijn niet alleen belangrijk als leidraad voor behandeling [10], maar breiden daarnaast het bereik van therapeutische mogelijkheden voor LCNEC en NETs uit [11]. Een onderzoek met *"antibody-drug conjugate"* (ADC) Rova-T toonde een totaal responspercentage van 13% in NEC's en NETs die DLL3 tot expressie brengen [12].

Studies met nieuwe medicatie of nieuwe doelwitten voor SCLC, zoals lurbinectedine plus de ATR-kinaseremmer berzosertib, de ADCs ABBV-706 en ABBV-011, multiple BiTes en CAR-T, bevatten nu patiënten met NETs en LCNEC, en zullen daardoor hopelijk het therapeutische arsenaal in alle NETs vergroten [13-19].

NSCLC MET EEN *K-RAS* MUTATIE

In het tweede deel van het proefschrift hebben we haalbaarheidsstudies uitgevoerd bij een cohort patiënten met gevorderd Kirsten rat sarcoom viraal oncogeen homoloog (*K-ras*) gemuteerd NSCLC. Momenteel wordt het PD-L1 eiwit gebruikt als biomarker om de respons op immuuntherapie bij NSCLC te voorspellen [20]. Toen we in 2015 met de studie begonnen, gebruikten we PD-L1 nog niet om therapiebeslissingen te nemen. Voor

een patiënt was het hebben van een NSCLC met een *K-ras*-mutatie de voorwaarde om deel te kunnen nemen aan de studie, mits aan de andere inclusiecriteria werd voldaan.

In **hoofdstuk 6** beschreven we de studie waarin we de klinische relevantie wilden beoordelen van het monitoren van circulerend tumor DNA (ctDNA) in bloedmonsters van patiënten met *K-ras*-gemuteerd NSCLC die werden behandeld met immuuntherapie. Het ultieme doel is vroegtijdige responsen te detecteren en te voorspellen voor langdurig effect op immuuntherapie. Alles bij elkaar was een gedaald aantal mutantkopieën, geschat met digitale druppel-PCR, geassocieerd met langere progressievrije overleving en totale overleving vergeleken met patiënten die verhoogde of stabiele ctDNA-niveaus toonden. CtDNA-dynamiek in combinatie met PD-L1-status is een veelbelovende, kosteneffectieve benadering om een langdurig effect, progressievrije en totale overleving, te monitoren bij patiënten met gevorderd NSCLC die worden behandeld met immuuntherapie. Het meten in de bloedsomloop van een enkele, van een tumor afkomstige moleculaire afwijking, verbetert de vroege herkenning van een aanhoudend klinisch effect en kan helpen bij het nemen van behandelbeslissingen.

Het monitoren van de ctDNA-dynamiek is een eenvoudig te gebruiken en een veelbelovend monitoringinstrument

In **hoofdstuk 7** onderzochten we het darmmicrobioom in een Nederlands cohort van 33 patiënten met een *K-ras*-gemuteerd NSCLC die behandeld werden met immuuntherapie. We vonden stammen (taxonomische rangen) en routes die verband houden met de respons op immuuntherapie en immuungerelateerde bijwerkingen in dit homogene cohort patiënten. We vonden een overlap in microbiële kenmerken van respons en behandelresistentie bij een cohort melanoompatiënten, wat duidt op gedeelde signalen tussen verschillende tumortypen. Het darmmicrobioom weerspiegelt mogelijk algemene mechanismen en kenmerken van het microbioom en lijkt daarmee tumoronafhankelijk.

Het darmmicrobioom weerspiegelt mogelijk algemene mechanismen en kenmerken van het microbioom en is daarom tumoronafhankelijk.

Detectie van *K-ras* mutaties

Tumorweefsel is de “gouden standaard” om *K-ras*-mutaties te detecteren, maar weefsel is niet altijd voorhanden en het afnemen van een bloedmonster bij de patiënt is minder invasief. Identificatie van *K-ras* G12C-mutaties in circulerend tumor-DNA was haalbaar en de concordantie tussen ctDNA en tumorweefsel was hoog, ongeveer 98% [20, 21]. De meest gelijktijdig voorkomende mutatie die werd gevonden was *STK11*. Detectie van *K-ras*-mutaties in plasma kan dienen als vervanging voor weefselmonsters, hoewel deze

regelmatig moeten worden gemeten en de testresultaten binnen een redelijk tijdsbestek beschikbaar moeten zijn om klinische beslissingen voor de patiënt te kunnen nemen [21, 22].

Met volledige exome-sequencing van NSCLC werd ontdekt dat meer factoren geassocieerd zijn met respons op immuuntherapie, zoals bijvoorbeeld *"Tumor Mutational Burden"* (TMB) [23]. Mutaties als *STK11*/leverkinase B1 (*LKB1*) en Kelch-like ECH-associated protein 1 (*KEAP1*) zijn niet geassocieerd met enig langdurig klinisch voordeel van immuuntherapie [24, 25]. De impact ervan precies is bij *K-ras*-gemuteerde NSCLC-patiënten is echter onbekend [26]. Wat de invloed is van al deze mutaties en genetische veranderingen moet verder worden onderzocht in prospectieve studies.

Doelgerichte behandeling van *K-ras* mutaties

De *K-ras* utatie was de afgelopen decennia moeilijk te behandelen [27]. Onlangs is kreeg de *K-ras* G12C-mutatie veel aandacht, omdat effectieve remming van *K-ras* G12C mogelijk bleek met sotorasib en adagrasib [28, 29]. In een groep voorbehandelde NSCLC patiënten met een *K-ras* G12C-mutatie toonde sotorasib toonde een totaal responspercentage van 37% tot 43% [28]. Bovendien was sotorasib beter dan docetaxel in fase 3 met verbeterde progressievrije overleving en totaal responspercentage [30]. Ondanks deze bevindingen variëren klinische uitkomsten van vroege ziekteprogressie bij 5 tot 16% van de patiënten tot duurzame effecten met een 2-jaars overlevingspercentage van 32,5% [31].

Het grootste cohort NSCLC-patiënten met een *K-ras* G12C-mutatie bestond uit 424 patiënten die werden behandeld met sotorasib of adagrasib [32]. Co-mutaties die werden gezien in de patiëntengroep met vroege progressie (< 3 maanden) waren *KEAP1*, *SMARCA4*, *CDKNA2A* en *STK11*. *TP53* was het meest frequent gemuteerde gen in het cohort, maar had geen correlatie met klinische resultaten. DNA-schadeherstelgenen (DDR) zoals we zagen in SCLC, *ATM*, *ATR* en *CHK1*, zorgden voor een hoger totaal responspercentage (52,2% versus 27,7%) en een significant langere progressievrije overleving (5,9 maanden vs. 4,6 maanden) met *K-ras* G12C vergeleken met DRR *wild type*. Er werd slechts numerieke overlevingswinst geboekt, zij het niet in statistisch significante mate.

Immuuntherapie was minder effectief in *STK11*- en *KEAP1*-gemuteerde longkanker [26]. STK11-verlies resulteerde in het uitschakelen van "*stimulator of interferon genes"* (STING) in KRAS, wat de immunogeniciteit in *K-ras*-gemuteerd NSCLC vermindert [33]. Bovendien was de *STK11*-mutatie geassocieerd met een gebrek aan PD-L1-expressie, verminderde tumor-infiltrerende cytotoxische CD8+ T-lymfocyten en resistentie tegen immuuntherapie bij patiënten met een *K-ras*-mutatie [24, 34]. Ondanks het feit dat

correlatie met een hoog TMB gezien werd, bleek *KEAP1* een negatief voorspellende biomarker voor de respons op immuuntherapie. Daarnaast werd een associatie gevonden met minder immuuninfiltraten die duiden op een *"cold tumor microenvironment*" [35].

In een cohort van 1261 patiënten met NSCLC werd *K-ras*-mutatie gedetecteerd bij 536 patiënten (42,5%), een *STK11*-mutatie bij 20,6% en *KEAP1*-mutatie bij 19,2% [36]. Bij ongeveer 10% werden meerdere gelijktijdig voorkomende mutaties gevonden. In de *K-ras*-groep hadden *STK11*- en *KEAP1*-mutaties een significant lagere PD-L1-expressie. TMB was hoger in de *STK11*- en *KEAP1*-groep. Daarnaast is de aanwezigheid van *STK11* en *KEAP1* voorspellend voor slechtere resultaten bij behandeling met chemotherapie. In deze studie wordt *KEAP1*, dat gelijktijdig voorkomt met *K-ras* G12C, echter geassocieerd met een slechte prognose en een inferieure uitkomst. Co-mutatie van *STK11* zonder een *KEAP1*-mutatie had geen invloed op het totaal responspercentage, progressievrije overleving of totale overleving *K-ras* G12C. Een onderzoek onder 330 patiënten vond *TP53* (42%), *STK11* (29%) en *KEAP1* (27%) [36]. Een significant kortere overleving werd gezien bij co-mutatie *KEAP1*, zowel bij behandeling met chemotherapie als bij immuuntherapie. De co-mutatiestatus van *STK11* en *TP53* was niet geassocieerd met overleving.

Of de aanwezigheid van *STK11*- en *KEAP1*-mutaties een slechtere uitkomst voorspelt bij immuuntherapie alleen of in combinatie met andere behandelingen, zoals doelgerichte therapie, chemotherapie of radiotherapie, zal op een prospectieve manier moeten worden geëvalueerd. Op dit moment zijn ze eerder prognostisch dan voorspellend voor een respons.

Andere *K-ras*-mutaties vertonen echter verschillende klinische uitkomsten, wat een andere onderliggende biologie suggereert [37]. *K-ras* G12D-mutaties kwamen bijvoorbeeld veel voor bij niet-rokers vergeleken met *K-ras* G12C. In deze groep ging behandeling met alleen immuuntherapie gepaard met een slechtere uitkomst, een totaal responspercentage van 15,8% versus 28,4% in de totale *K-ras*-gemuteerde groep. Behandeling met chemo-immuuntherapie was in deze groep superieur. Nieuwe klinische onderzoeken zullen moeten leiden tot stratificatie van verschillende *K-ras*-mutaties of gelijktijdig voorkomende mutaties.

Met de onderzoeken die in dit proefschrift worden beschreven, hebben we een diepgaander inzicht kunnen verwerven in nieuwe biomarkers in longtumoren. Biomarkers zijn dus van cruciaal belang bij het selecteren en stratificeren van patiënten voor specifieke behandelingen.

WEEFSEL

Ondertussen blijft het verkrijgen van weefsel van het grootste belang. Niet alleen om een longnodule te diagnosticeren, maar ook om een keuze te maken tussen de nieuwe behandelmogelijkheden die de laatste tijd beschikbaar zijn gekomen. Diagnostische technieken voor het beoordelen van longlesies blijven een uitdaging. Met nieuwere bronchoscopietechnieken, zoals virtuele navigatiebronchoscopie, om weefsel te verkrijgen van moeilijk bereikbare afwijkingen in de long, is het nu mogelijk om volledige moleculaire analyses uit te voeren en een passende behandeling aan te bieden. Of, in het geval van progressieve ziekte van de groeiende afwijking, stellen deze technieken ons in staat nieuw weefsel te verkrijgen en resistentiemechanismen te beoordelen voor een andere therapiekeuze. Voldoende weefsel is belangrijk voor het uitvoeren van een goede moleculaire analyse in de ontwikkeling naar precisiegeneeskunde.

In **hoofdstuk 8** presenteerden we een *'Les van de maand'* over een oudere patiënt met chronische myelomonocytaire leukemie (CMML), bij wie longadenocarcinoom werd vastgesteld. In de longbiopsie werden verschillende mutaties gevonden: een *PIK3CA*-mutatie, een niet-gekarakteriseerde *MET*-frameshift-mutatie en een *IDH2*-mutatie. Deze laatste is ontstaan door tumor-infiltrerende CMML. De twee andere mutaties zijn afkomstig van de longkanker. De *PIK3CA*-mutatie is een bekende driver-mutatie bij longkanker; de *MET*-frameshift-mutatie bleek het *"skippen"* van MET exon 14 te induceren, welke met succes werd behandeld met crizotinib. Dit geval illustreert dat ogenschijnlijk onverwachte mutaties het gevolg kunnen zijn van een infiltrerende tweede maligniteit, die mogelijk niet door histomorfologie alleen wordt herkend. Indien een onverwachte genetische variant wordt gedetecteerd in de moleculaire analyse van solide maligniteiten, moet rekening worden gehouden met de aanwezigheid van een tweede maligniteit.

In **hoofdstuk 9** rapporteerde we de resultaten van – NAVIGATOR –, een monocentrum, prospectieve, observationele studie uitgevoerd bij patiënten die een virtuele bronchoscopie-navigatie (VBN) procedure ondergingen om een longnodule te beoordelen. We presenteerden een cohort van de eerste 35 opeenvolgende patiënten die aan ons programma deelnamen. De longnodules waren moeilijk te bereiken en werden niet toegankelijk geacht met een CT-geleide transthoracale biopsie, een conventionele bronchoscopie of met een endobronchiale echografie (EBUS). De diagnostische opbrengst was 77% en bleek afhankelijk van de afmeting van de nodule en de gekozen route. Complicaties waren zeldzaam en beheersbaar. Het voorkómen van meer invasieve procedures met een hoger complicatierisico zoals chirurgie of transthoracale puncties is gunstig voor patiënten. Met het uitvoeren van VBN met een diagnostische opbrengst

van 77% bij longnodules waarvan anders geen diagnose zou worden gesteld, maakte dat we bij tweederde van de patiëntenpopulatie het behandelplan konden aanpassen naar een meer gerichte behandeling.

Nieuwe benaderingen om tumorweefsel te beoordelen bij (vermoedelijke) longkankerpatiënten zijn mogelijk.

In het snel evoluerende veld van interventiebronchoscopie hebben we in ons NAVIGATOR-onderzoek geconcludeerd dat we nog steeds de diagnostische nauwkeurigheid en aanvullende lokalisatiebevestiging van de positie van de longnodule moeten verbeteren. Toevoeging van radiale EBUS en cone-beam CT-scan tijdens de procedure wordt sterk aanbevolen [38]. Niet alleen om complicaties, zoals bloedingen en pneumothorax, te voorkomen, maar belangrijker nog: om de diagnostische opbrengst van de ingreep te verhogen door een betere plaatsbepaling voorafgaand aan het nemen van de biopten. Daarnaast zijn betere selectiecriteria nodig om patiënten te kunnen toewijzen aan het diagnostische instrument dat het meest geschikt is om weefsel te verkrijgen, namelijk de VBN of een CT-geleide transthoracale biopsie. Correlatie met gedetailleerde klinische gegevens zal helpen om meer inzicht te verkrijgen in dit vraagstuk. Vergelijking van gegevens van uitgebreide aantallen procedures in NAVIGATOR met bestaande gegevens van patiënten die CT-geleide transthoracale puncties hebben ondergaan in ons centrum in de afgelopen 5 jaar kan de ontwikkeling van selectiecriteria vergemakkelijken om patiënten toe te wijzen aan de veiligste procedure met a priori de hoogste diagnostische opbrengst.

Samen met andere expertisecentra in Nederland bouwen we momenteel een database met gegevens over patiënten en procedures om navigatiebronchoscopie een solide plek te geven in het diagnostisch arsenaal van een longnodule.

Virtuele navigatiebronchoscopie

Virtuele bronchoscopie heeft voordelen bij het verstrekken van informatie over de luchtwegen, bloedvaten en de pleura. De plannings-CT-scan is van het grootste belang omdat de virtuele omgeving ervan afhankelijk is. De procedurele problemen die mogelijk de nauwkeurige planning van de virtuele paden naar de nodule belemmerden, waren de resolutie van de laesie op de pre-procedurele CT-scan en fysieke blokkades zoals slijmimpactie in kleinere luchtwegen [39]. Bovendien is de zogenaamde CT-lichaamsdivergentie een groot obstakel voor succesvolle navigatie [39]. Dit betekent dat pre-procedurele longvolumes op de geplande CT drastisch kunnen verschillen van procedurele longvolumes onder algehele anesthesie bij de geïntubeerde patiënt [40]. Immers, patiënten kunnen in wakkere situatie veel dieper inademen dan de inspiratie die tijdens algehele anesthesie bij een geïntubeerde patiënt bereikt kan worden. Vooral

in de onderste longkwabben kan de overeenkomst vergeleken met de planning laag zijn. Longnodules kunnen zeker 2 cm in positie bewegen [41], er kunnen mismatches optreden als gevolg van een inadequate positionering van de patiënt op de tafel in vergelijking met de CT-scan, en er kan een verschil zijn tussen door de patiënt getriggerde diepe inhalatie tijdens de scan en de ademhaling onder narcose tijdens de procedure [40]. Er zijn effectievere beeldvormingstechnieken beschikbaar, zoals de cone-beam CT met lichaamsvormdetectie, om ademhalingsproblemen en CT-lichaamsdivergentie onder controle te brengen en de diagnostische nauwkeurigheid te verbeteren [42].

Robotgestuurde bronchoscopie

Om de beperkingen op het gebied van navigatie en diagnostische opbrengst te overwinnen, zijn verschillende robotgestuurde bronchoscopieplatforms (RAB) ontwikkeld. Twee platforms voor RAB-navigatie hebben goedkeuring gekregen van de Food and Drug Administration (FDA) en zijn momenteel beschikbaar in de Verenigde Staten [43]. In Europa is nog geen toestemming voor patiëntenzorg ontvangen, waardoor platforms op dit moment alleen beschikbaar zijn voor onderzoeksdoeleinden. Het eerste, het Monarch RAB-platform (Auris Health, Inc., Redwood City, CA, VS) is gebaseerd op elektromagnetische navigatietechnologie (EMN) [44]. Het Ion Robotic-Assisted Endoluminal Platform (Intuitive Surgical, Inc., Sunnyvale, CA, VS) is gebaseerd op vormdetectietechnologie, waarbij gebruik wordt gemaakt van glasvezelbuigsensoren in de katheter zelf om de oriëntatie te behouden [45]. Een derde platform, het Galaxy System™ (Noah Medical, San Carlos, CA, VS) heeft in 2023 goedkeuring van de FDA verkregen, biedt EMN en digitale tomosynthesebegeleiding naast een wegwerp-bronchoscoop [43].

Het belangrijkste voordeel van RAB-navigatie is het behoud van een statische positie om onder continue visualisatie naar zeer kleine perifere luchtwegen te navigeren [46]. Het navigatiesucces was 88,6% met een diagnostische opbrengst van 69,1% in het eerste onderzoek van EMN RAB [47]. Veilige katheterpositionering in de nabijheid van de laesie was haalbaar en veilig (in Ion RAB) [48]. De nabijheid van de katheter tot de doellaesie was de sterkste voorspeller voor een diagnose, onafhankelijk van de aanwezigheid van een bronchusteken of een concentrisch radiaal EBUS-beeld [49]. Toevoeging van de cone-beam CT ter bevestiging van de juiste navigatie en tool-in-laesie tijdens biopsiemonsters verbeterde de diagnostische opbrengst tot 83% [50]. Dit benadrukt de vraag of de diagnostische opbrengst het juiste primaire eindpunt is, en of diagnostische nauwkeurigheid een betere uitkomstmaat is, die follow-up mogelijk maakt [51]. Over het geheel genomen zijn RAB-navigatie naar de laesie met een bronchusteken, bevestiging van de juiste locatie van het biopteur en het verkrijgen van voldoende weefsel voor diagnose alle haalbaar [52]. Stabiliteit en precisie zijn de sleutelwoorden om perifere

laesies te bereiken die anders niet gediagnosticeerd zouden kunnen worden met behulp van een door robots ondersteund bronchoscopieplatform.

Ook op RAB-platforms blijft de divergentie tussen CT-scan en lichaam een groot obstakel voor succesvolle navigatie [42]. Toegevoegde beeldvorming, zoals doorlichting of cone-beam CT-scan, kan dit probleem oplossen. Om de lokalisatiecontrole te verbeteren, werd de O-arm CT-scan toegevoegd [53]. Om deze nadelen te ondervangen, blijkt het Ion-platform met vormgevoelig vermogen op dit moment het meest praktisch.

Het nadeel van robotsystemen is dat ze duur zijn, terwijl publicaties over initiële kosten, onderhoud en procedurele hulpmiddelen niet beschikbaar zijn. Prospectieve onderlinge vergelijkende studies van RAB en EMN zijn nog niet uitgevoerd, en retrospectief is de diagnostische opbrengst ongeveer vergelijkbaar [54]. Informatie over de kostenontwikkeling en de vergoedingsopties voor robotsystemen moet worden verkregen voordat ze in de dagelijkse patiëntenzorg worden geïntroduceerd. Aan de andere kant staat buiten kijf dat longnodules in de toekomst met meer precisie moeten worden bereikt en gediagnosticeerd.

Bronchoscopie in de toekomst

Naarmate de tijd verstrijkt, zullen de navigatie-bronchoscopieprocedures toegang bieden tot nieuwe geavanceerde beeldvormingstechnieken, zoals confocale laser-endomicoscopie en fluorescentie-moleculaire endoscopie, die de identificatie van de laesie tijdens de endoscopische procedure zullen vergemakkelijken [55]. Beide technieken zouden kunnen worden gecombineerd met laser-versterkte fluorescentie-gelabelde tracerdetectie om de gevoeligheid voor medicatie te beoordelen [56, 57]. Tenslotte zal ook in de toekomst een verbeterde lokalisatie van de nodule nodig zijn om lokale ablatieve therapieën veilig toe te kunnen passen met minimale schade aan gezond longweefsel. Behandeling van de kwaadaardige nodule met microgolfablatie onder begeleiding van een navigatiebronchoscopie of RAB-geleide biopsie zal het onderwerp zijn van toekomstige studies [58, 59].

Uiteindelijk verwachten we dat de ontwikkeling en combinatie van deze technieken de weg zal vrijmaken voor een *“one-stop-shop”*-aanpak, met snelle evaluatie ter plaatse met beeldvorming en een biopsie, gevolgd door lokale behandeling van de kwaadaardige longnodule.

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Appendices

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Dankwoord

Curriculum Vitae

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DANKWOORD

“Wat is belangrijker,” vroeg Grote Panda, “de reis of de bestemming?”
“Het gezelschap,“ zei Kleine Draak.

(Uit: Grote Panda & Kleine Draak – James Norbury)

Promoveren was voor mij een internationaal, multicentrisch en longitudinaal project. Het lijkt bijna op een fase-1 studie. In deze reis door de tijd heb ik veel mensen ontmoet. Uiteraard patiënten en hun naasten die mij toevertrouwden om passant te zijn in een periode in het leven waarin rouw en ontzetting dichtbij zijn. Juist voor deze mensen hoop ik dat dit proefschrift een aanzet is tot meer duidelijkheid over hun ziekte en de behandeling daarvan. Dank voor jullie moed en volharding!

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CURRICULUM VITAE

Birgitta Ingrid Hiddinga werd geboren in Groningen. Na het behalen van het VWO diploma aan de Rijksscholengemeenschap Coevorden keerde ze terug naar Groningen voor haar studie geneeskunde. Eén coschap – interne geneeskunde – was in het kader van Erasmusuitwisseling in het Universitair Ziekenhuis Antwerpen. Na haar diplomering deed ze een breed palet aan klinische vaardigheden op in onder andere in de kindergeneeskunde en jeugdgezondheidzorg. Tevens was ze teamleider van een docententeam voor de deeltijdopleiding doktersassistent aan het Deltion College in Zwolle. Ze gaf daar anatomie, fysiologie en pathologie en was stagebegeleider. Toen het ziekenhuis trok, begon ze als ANIOS interne geneeskunde in Enschede, vanwaar ze overstapte naar de Longziekten in Medisch Spectrum Twente. Hier rondde ze haar opleiding af. Aansluitende verhuisde ze naar België voor een fellowship Thoracale Oncologie in Universitair Ziekenhuis Gent onder leiding van Jan van Meerbeeck en Veerle Surmont. Dit resulteerde in een aanstelling als staflid Thoracale Oncologie in Universitair Ziekenhuis Antwerpen van ruim 5 jaar. Sindsdien is zij werkzaam als longarts met aandachtsgebied longoncologie in het Universitair Medisch Centrum Groningen.