Universitat Autònoma de Barcelona

HETEROGENEITY OF BIOMARKER EXPRESSION IN NON-SMALL CELL LUNG CANCER

David Casadevall Aguilar

Universitat Autònoma de Barcelona
Facultat de Medicina
Departament de Medicina

HETEROGENEITY OF BIOMARKER EXPRESSION IN NON-SMALL CELL LUNG CANCER

PhD thesis presented by:

David Casadevall Aguilar

Thesis Directors:

Edurne Arriola Aperribay

Joan Albanell Mestres

Thesis Tutor:

Adolfo Díez Pérez

Doctoral Program in Medicine

September 2017

Als meus pares, Jordi i Eli, i al meu germà Jan, per ser incondicionals.

A l'Erika, per acompanyar-me i per ser la súper-mare de la Violeta.

A la Laura i la Marian, perquè un dia vencerem aquesta malaltia.

"The crab is the wrong symbol for cancer. They should have used a chimera, a monster in Greek mythology that has a goat's body, a serpent's tail, and a lion's head, and is often shown breathing fire. It is a much fiercer and more dangerous animal than a crab."

Dr. Paul Calabresi (1930-2003)

INDEX

Acknowledgements

Només puc tenir paraules d'agraïment cap a l'Edurne Arriola. Et vull donar les gràcies per creure en mi des del principi i donar-me forces i empenta per créixer professionalment. Sense tu no hi hauria tesi. Ets un exemple d'eficiència i organització i una gran mentora.

També li estic molt agraït al Joan Albanell. Vas ser el meu primer professor d'oncologia a la facultat i posteriorment has esdevingut una referència com a oncòleg i com a investigador. T'agraeixo els teus consells, sempre valuosos, i el teu suport per seguir endavant en el que m'apassiona.

A la Lara Pijuan, gràcies per la teva saviesa i la tevaa (infinita) paciència davant del microscopi. Gràcies per contestar els whatsapps al minut i per deixar-me el teu despatx en hores mortes. Sense el teu "expertise" en patologia digital no haguéssim quantificat ni un limfòcit.

Al Sergi Clavé, gràcies per la teva bona disposició. Sense la teva bona feina la tesi tindria menys valor. Òbviament aquí s'hi ha d'afegir a la Marta Salido, la millor cantant del servei de patologia i jo crec que el somriure més perenne i contagiós de tota la planta -1 de l'hospital. Gràcies als dos per la vostra actitud sempre col·laboradora, així com per les bones estones tan dins com fora de l'hospital.

Al Javi Gimeno, per la seva implicació i compromís durant l'època en que vam treballar junts. El millor professor del multi-capçal i l'amo de l'H-score.

A la Marta Lorenzo i la Silvia Menéndez, reines de la parafina i artífexs dels TMA.

A l'Álvaro Taus, per les seves solucions sempre pràctiques i per la seva ajuda amb la farragosa burocràcia.

Al Max Hardy, a l'Andrés Pérez i a l'Albert Puig, per la seva revisió del text i els seus "inputs".

A la Míriam Arumí, la meva co-R, per sempre tenir un sí a la boca i voluntat d'ajudar. Tens l'únic somrirure que podria entrar a concurs amb el de la Marta. Et trobo a faltar.

A la Joana Vidal, per ajudar-me a alliberar l'estrès (paradoxalment molts cops amb cafès) i compartir alegries i penes del dia a dia.

Al Fran Sánchez, per ser el millor company de despatx i de la sala de revelat. Per la seva paciència quan m'ha tocat pipetejar i calcular els comptes de la Neubauer. Ell assegura que els Westerns em surten prou bé, però jo no ho acabo de veure...

A la Noelia Tarazona, per ser com és i engrescar a tothom amb la seva passió i entusiasme per la recerca.

A la resta de la gent que ha format part del servei d'Oncologia Mèdica de l'Hospital del Mar durant la meva residència, especialment a les tutores de residents Sònia Servitja i Marta Guix, però també a l'Ignasi Tusquets, la Maria Martínez, l'Ainhoa Hernández, la Laura Visa, l'Alejo Rodríguez-Vida, la Tamara Martos, la Laia Garrigós, la Iria González i la Isabel López.

A tot l'equip d'infermeria, auxil·liars i les administratives de planta i d'Hospital de Dia, per la seva ajuda en el dia a dia i pel seu tracte amb els pacients.

A les companyes d'assajos clínics, sempre veloces amb els mails i peces essencials en el dia a dia dels dispensaris, la Susanna Galtés, la Marta Macià, la Clara Carreras, l'Andrea Edo, la Gemma Martínez, la Roser Correa i la Bàrbara Pons.

Als residents, presents i passats: Eli Mompradé, Laia Espasa, Ana Luque, Noelia Vilariño, Maria Pinto-Machado, Anna Mateo, Pedro Rocha, Mayra Orrillo, Àlex Corbera, Sílvia Ribas, Laura Moliner, Josep Sabaté, Alejandro Ríos y Xavier Monzonis.

Als membres del laboratori del Programa de Recerca en Càncer, encapçalat per l'Ana Rovira: Oriol Arpí, Ali Sabbaghi, Anna Hernández, Aida Sampera, Cristina Guardia, Jennifer Linares, Alexandre Calon i Sílvia Geeraerd.

A l'Àlex, per la seva amistat "de guàrdia", serena i divertida alhora. Ets el paradigma de que els amics són la família que triem. I parlant de famílies postisses, aquí he de destacar l'inestimable privilegi que ha suposat gaudir de l'amistat dels meus companys d'aventures, l'Albert, l'Andrés, els Danis, el Guille, el Mar, el Nachete i el Pablo. Gràcies a vosaltres la residència ha estat un compendi d'experiències inoblidables.

A la Laia, per ser una amiga des que tinc memòria i la germana que mai he tingut (no offense, Jan). Portbou no seria el mateix sense tu. Espero seguir fent bufandes molt de temps.

Vull agrair a la meva família i especialment als meus pares, el seu recolzament sense matisos. Són els que m'han donat l'oportunitat d'estudiar el que he volgut i han fet el possible per brindar-me l'entorn per fer-ho. Sense ells, sens dubte, no seria on sóc. Finalment, agrair també a l'Erika i a la Violeta, que són la peça fonamental del meu present i la clau del meu futur. Em doneu la força per aixecar-me cada matí i sou la raó per tornar a casa cada vespre.

Summary

The success of precision medicine in oncology is dependent to a large extent on an adequate selection of patients who will receive targeted therapies aimed at specific molecular traits of their tumor. In order to be able conduct such patient selection, predictive biomarkers that can inform therapeutic decisions are essential.

MET and PD-L1 are two relevant membrane receptors for non-small cell lung cancer (NSCLC) biology. MET is an oncogene the activation of which is involved in multiple pro-tumorigenic processes such as cell proliferation, motility and invasion. PD-L1 is a key molecule that acts during the immune response, and its overexpression in tumors is thought to mediate the ability of tumor cells to avoid immune cell recognition and destruction. Currently, there are specific therapies directed against these molecules. The most commonly used strategy to select the patients that will benefit from such drugs is the analysis of the expression of both molecules in tumor tissue. However, the value of MET and PD-L1 as predictive biomarkers and the method by which it should be determined is a subject of debate.

Recent studies have detected a high degree of genomic heterogeneity in NSCLC tumor samples. This heterogeneity could significantly affect biomarker-based patient classification especially in the case of NSCLC, since biomarker studies are usually performed in small biopsies or cytology samples obtained through minimally invasive techniques. The main objective of the work presented in this thesis is to study the heterogeneity of the expression of MET and PD-L1 in NSCLC samples.

For this purpose, we have analyzed tumor samples from NSCLC patients that had undergone surgical treatment at Hospital del Mar. Of each tumor, we have selected multiple geographically separate areas, which we analyzed independently. In the study evaluating MET, we selected four tumor areas per patient, while in the study evaluating PD-L1 we selected two areas. In each tumor area, we measured the expression MET and PD-L1 using immunohistochemical and fluorescence in situ hybridization methods (FISH). Finally, we compared the expression of MET and PD-L1 in different tumor areas.

Regarding MET, we have found discordances between different tumor areas in 20-40% of cases using immunohistochemistry and in 25-50% of cases using FISH. Regarding PD-L1, this discrepancy was greater if we evaluated PD-L1 expression in tumor infiltrating lymphocytes (17-27%) than if we did so only in tumor cells (10-19%). Moreover, 36% of the cases with amplification of the gene coding for PD-L1 determined by FISH presented gene amplification only in one of the two areas analyzed.

Overall, our results suggest that the expression of both biomarkers is heterogeneous, whether measured by immunohistochemistry or by FISH. This heterogeneity can have a potential impact on the classification of tumors based on the expression of biomarkers and, therefore, could represent a hurdle for the development of targeted therapies for NSCLC patients.

Resum

L'èxit de de la medicina de precisió en oncologia depèn, en gran mesura, d'una adequada selecció dels pacients que rebran teràpies dirigides contra dianes específiques del seu tumor. Per poder seleccionar els pacients, és indispensable disposar de biomarcadors amb valor predictiu que informin les decisions terapèutiques.

MET i PD-L1 són dos receptors de membrana rellevants en la biologia del carcinoma pulmonar no microcític (CPNM). *MET* és un oncogen i l'activació de la seva via es troba relacionada amb múltiples processos pro-tumorals com són la proliferació i la motilitat cel·lulars, així com la invasió d'estructures veïnes. PD-L1 és una molècula clau en la resposta immunitàries, i la seva sobre-expressió en els tumors està relacionada amb la capacitat de les cèl·lules tumorals d'evitar el seu reconeixement i destrucció per part del sistema immunitari. Actualment, existeixen teràpies específiques dirigides contra aquestes molècules. L'estratègia més emprada per seleccionar els pacients que se'n poden beneficiar és la determinació de l'expressió d'ambdues molècules en teixit tumoral. Tanmateix, el valor de MET i de PD-L1 com a biomarcadors predictius i el mètode pel qual s'han de determinar és subjecte de debat.

Estudis recents han detectat un alt grau d'heterogeneïtat genòmica en mostres tumorals en CPNM. Aquesta heterogeneïtat podria afectar de forma rellevant la classificació de pacients basada en l'expressió de biomarcadors. A més, aquest fet seria especialment rellevant en el cas del CPNM, ja que l'estudi de biomarcadors es fa generalment en mostres petites de teixit, provinents de biòpsies o citologies obtingudes mitjançant tècniques mínimament invasives. L'objectiu principal dels treballs presentats en aquesta tesi és estudiar l'heterogeneïtat de l'expressió de MET i PD-L1 en mostres de CPNM.

Amb aquesta finalitat, hem analitzat mostres tumorals procedents de pacients tractats quirúrgicament de CPNM a l'Hospital del Mar. De cada tumor, hem seleccionat múltiples àrees geogràficament separades, les quals hem analitzat de forma independent. En l'estudi en que hem avaluat MET hem seleccionat quatre àrees per cada pacient, mentre que en l'estudi de PD-L1 n'hem seleccionat dues. En cada àrea tumoral, hem mesurat l'expressió de MET i de PD-L1 mitjançant mètodes d'immunohistoquímica i d'hibridació in situ fluorescent (FISH). Finalment, hem comparat l'expressió de MET i de PD-L1 entre diferents àrees tumorals.

En el cas de MET, hem trobat discordances entre diferents àrees tumorals en un 20-40% per immunohistoquímica i en un 25-50% per FISH. En el cas de PD-L1, aquesta discordança ha estat major si es valora només l'expressió en limfòcits infiltrants de tumor (17-27%) que si es valora en cèl·lules tumorals (10-19%). A més, un 36% dels casos amb amplificació del gen que codifica PD-L1 determinada per FISH presenten aquesta amplificació només en una de les dues àrees analitzades.

En conjunt, els nostres resultats suggereixen que l'expressió d'ambdós biomarcadors és heterogènia, tant si es mesura mitjançant immunohistoqumímica com mitjançant FISH. Aquesta heterogeneïtat pot tenir un impacte potencial en la classificació de tumors basada en l'expressió de biomarcadors i per tant, pot suposar una dificultat afegida a l'hora de desenvolupar teràpies dirigides per pacients amb CPNM.

ABBREVIATIONS

ADC Adenocarcinoma

ALK Anaplastic Lymphoma Receptor Tyrosine Kinase

CEP7 Chromosome enumeration probe 7

EGFR Epidermal Growth Factor Receptor

FISH Fluorescence in situ hybridization

IFN-γ Interferon gamma

IHC Immunohistochemistry

ITH Intratumor heterogeneity

MET MET Proto-Oncogene

NSCLC Non-small cell lung cancer

OS Overall survival

PD-1 Programmed Cell Death Protein 1

PD-L1 Programmed Death Ligand 1

ROS1 ROS Proto-Oncogene 1

SCC Squamous cell carcinoma

UCCC University of Colorado Cancer Center

1. INTRODUCTION

1.1. Lung cancer

1.1.1. Epidemiology

Lung cancer is the second most incident malignancy and the first cancer-related cause of death in both sexes. This makes lung cancer a major health problem, accounting for 13% of all new cancer cases and for 19% of cancer-related deaths worldwide[1]. Lung cancer incidence is currently 5- to 7-fold higher in highly developed countries than in low developed countries, and the largest differences are observed in women[2]. Furthermore, it harbors a dismal prognosis, with five-year relative survival rates in Western countries that ranges from 10 to 15%[2]. In Spain, according to estimates by the 2012 GLOBOCAN project[1], lung cancer was the third most commonly diagnosed malignancy after colorectal and prostate cancer, and represented the most lethal cancer in both sexes, accounting for 26,715 new cases and 21,118 deaths. The Spanish Network of Cancer Registries (REDECAN) reported a total of 28,347 estimated new lung cancer diagnoses in 2015[3]. With respect to the 2012 report, new lung cancer cases increased in both sexes, but this increase was proportionally higher in women (16.5% vs. 3% in men)[2,3].

1.1.1. Overview of lung cancer diagnosis and staging

Unfortunately, lung cancer is usually asymptomatic until it reaches an advanced stage. Thus, lung cancer diagnoses occur when the disease has already spread in >50% of cases[4]. When lung cancer is suspected, the process of diagnosis and staging should be carried out by a multidisciplinary tumor board that involves medical and radiation oncologists, pulmonologists, thoracic surgeons, as well as specialized pathologists, radiologists, and nuclear medicine physicians. The first goal of the tumor board must be to establish a definitive histological diagnosis and to precisely determine the disease stage[5]. Until not long ago, staging of lung cancer has been performed according to the 7th edition of the International Association for the Study of Lung Cancer (IASCL) / Union Internationale Contre le Cancer (UICC) classification[6]. More recently, a proposal for the 8th edition of the IASCL/UICC classification has been published[7] and is being implemented since January 2017.

For adequate disease staging, a spiral contrasted CT scan is required. In patients in whom mediastinal disease is suspected, studies may be complemented by positron-emission tomography (PET) or PET-CT[8,9]. To obtain histological confirmation of lung cancer, minimally invasive endoscopic procedures procedures should be first attempted. If the primary lesion is peripheral and thus inaccessible by bronchoscopy, CT- or ultrasound-guided transthoracic biopsy is recommended[10–12]. Lastly, if the patient presents with advanced disease, ultrasound-

guided biopsy of accessible metastatic sites may also be considered. Both diagnostic strategies generally provide small samples, a few millimeters in size, representing a sole area of the whole tumor.

1.1.2. Overview of NSCLC treatment

The treatment strategy for each lung cancer patient should be carefully evaluated in the context of an expert multidisciplinary tumor board and should integrate clinical, radiological, histopathological, and molecular information. The optimal treatment strategy should be decided after a detailed discussion between the informed patient and the treating physician[5,10,13,14].

Treatment of patients with localized disease is essentially based on the presence or absence of mediastinal disease. Early stage lung cancers (stages I and II), in which there is no evidence of mediastinal involvement, should be considered for surgical resection. The preferred surgical approach is a lobectomy, although limited resections can be considered in some cases involving small (≤2cm) non-solid primary lesions[15] or in patients with moderate/severe pulmonary dysfunction[16]. Stage III disease (i.e. presence of mediastinal involvement) represents a highly diverse set of clinical settings depending on the extent of disease and patient charactersitics. Neoadjuvant chemotherapy or chemoradiotherapy are valid strategies, as well as definitive concurrent chemoradiotherapy, depending on the context[17–22]. Also, if mediastinal involvement is detected incidentally during pathological evaluation of the surgical specimen, adjuvant chemotherapy may be offered[23,24].

Stage IV lung cancer harbors a dismal prognosis despite systemic treatment. For all patients with advanced lung cancer, smoking cessation should be encouraged and adequate palliative care should be offered, since both measures are clearly beneficial[25,26]. In this context, surgery and radiotherapy may be indicated, but only to prevent clinically relevant complications or to alleviate symptoms[13]. Until recently, the vast majority of advanced NSCLC patients were treated with systemic combination chemotherapy, which consisted of platinum-based doublets[27–31]. Nowadays, however, systemic treatment is guided by various molecular and immunohistochemical assessments in the diagnostic biopsy. Thus, adequate histological and molecular classification is of paramount importance for determining the optimal therapeutic strategy in advanced NSCLC.

1.2. NSCLC classification and therapeutic implications

1.2.1. Histological classification of NSCLC

The first histological classification of lung cancer was published by the World Health Organization in 1967 and has been updated several times since then[32–34]. Currently, lung cancer is classified according to the 2015 World Health Organization (WHO) classification of lung tumors[35]. Until approximately eight years ago, histological subtyping of lung cancer had no clinical or therapeutic relevance. Today, however, accurate histopathological and molecular classification has direct prognostic and predictive implications and, as mentioned above, is a crucial factor for therapeutic decision-making.

Since the 2011 update[36], the classification of lung cancer takes into account the fact that 60-70% of patients are diagnosed at an advanced disease stage and that, therefore, tumor tissue availability for diagnostic purposes is frequently limited[37]. Thus, specific criteria have been developed for the analysis of small biopsy or cytology samples. Accordingly, one of the mainstays of the latest classifications is the implementation of an efficient workflow that allows for immunohistochemical and molecular analysis while maximizing tissue preservation.

Non-small cell lung cancer comprises around 85% of all lung cancers, among which 40-50% are adenocarcinomas (ADC). Squamous cell carcinomas (SCC) account for up to 30% of NSCLC[38]. The remaining cases can be classified into large cell carcinoma or rarer subtypes such as sarcomatoid, enteric or adenosquamous carcinomas. Well-differentiated tumors can be classified based only on Hematoxylin/Eosin (H&E) staining. However, specific immunohistochemical stains are indicated to increase diagnostic accuracy in morphologically equivocal samples. Thus, markers such as TTF-1, p40 and cytokeratins 5, 6 and 7 can help distinguish between ADC and SCC. This seemingly elementary histological distinction will already be informative of potential treatment strategies. For example, neither pemetrexed nor bevacizumab should be offered to patients with squamous-cell histology, due to a lack of efficacy and high rates of severe complications, respectively[39–42]. Instead, they may be offed other platinum-accompanying agents such as gemcitabine, vinorelbine or a taxane can be offered[13,43].

Both ADC and SCC can be further sub-classified into distinct subtypes. Invasive ADCs can present with different growth patterns such as lepidic, papillary, acinar, solid, or micropapllary (Figure 1)[35,36]. Two or more of these patterns often coexist within the same tumor. Pathological assessment of invasive adenocarcinomas needs to account for each of these

growth patterns and quantify them in 5% increments. The final pathology report should also state the predominant growth pattern[35,36]. This subclassification of ADC samples may bear direct clinical significance. Indeed, acinar and lepidic growth patterns have been associated with a good prognosis while solid and micropapillary have been postulated as adverse prognostic factors[44,45]. Furthermore, a predominantly solid pattern may be predictive of adjuvant chemotherapy benefit[45,46]. In contrast to ADC, lung SCC is generally considered more homogeneous in growth and should be classified into keratinizing, non-keratinizing and basaloid. However, histological subclassification of SCC specimens has not yet shown significant clinical implications. Ultimately, the distinction between ADC and SCC histology will guide the screening for further histological or molecular alterations that are amenable to specific targeted therapies. Currently, testing for such distinctive alterations is only routinely recommended for ADCs and for patients SCC with minimal or absence of smoking history[13,14,35].

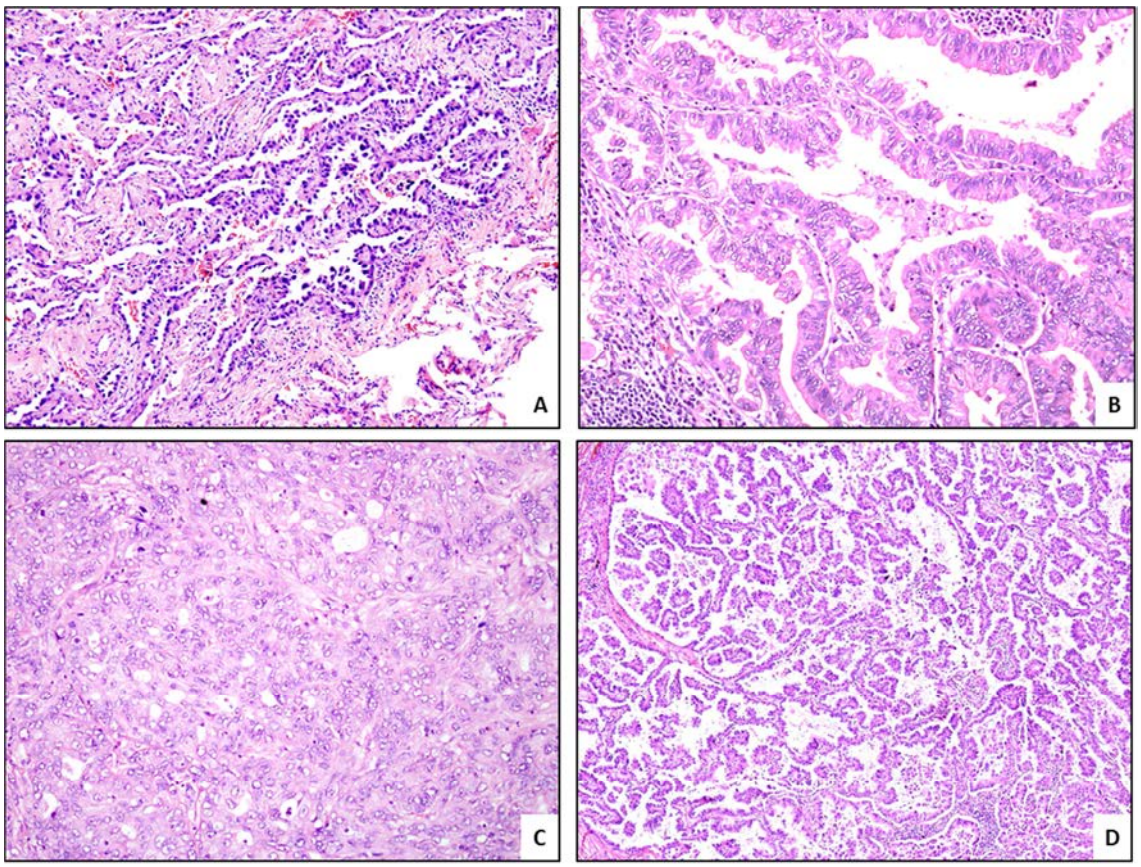


Figure 1. Examples of different growth patterns in lung adenocarcinoma. **A:** lepidic, **B:** acinar, **C:** solid, **D:** papillary. Courtesy of Dr. Lara Pijuan.

1.2.2. Molecular classification and targeted therapy in NSCLC

Similarly to what happened in other cancer types such as breast cancer, chronic myeloid leukemia or melanoma[47–49], the emergence of targeted therapies has yielded significant results in NSCLC. Targeted therapies rely on the identification of specific molecular traits that are relevant for tumor biology. These molecular traits are usually present only in a subset of patients with a given cancer type. Appropriate patient selection for targeted therapy ideally aims at identifying those patients that will benefit from a specific treatment while excluding patients that would present no or poor response, thus avoiding ineffective treatments and unnecessary toxicities for the latter. Furthermore, by narrowing the patient population that will receive a given treatment, treatment- and toxicity-derived costs are avoided, which is critical for the economical sustainability of anti-cancer treatments.

The first successfully targeted molecular alteration in NSCLC was the constitutive activation of the Epidermal Growth Factor Receptor (EGFR) due to specific mutations in the genetic region of its tyrosine kinase domain. Such mutations occur almost exclusively in ADCs and confer exquisite sensitivity to specific EGFR tyrosine kinase inhibitors[50]. In the subset of ADC patients harboring *EGFR* mutations (approximately 12-16% in our population), treatment with specific inhibitors such as erlotinib, gefitinib or afatinib has proven to be clearly superior to treatment with standard chemotherapy[51–53].

Following the discovery of *EGFR* mutations, another relevant molecular alteration was identified, which is the occurrence of recurrent chromosomal rearrangements that involved the Anaplastic Lymphoma Tyrosine Kinase Receptor (*ALK*) gene and resulted in increased proliferation and malignant transformation in around 5% of our patients[54]. Similarly to what was observed with EGFR inhibitors in *EGFR*-mutant NSCLC, it was soon proven that the ALK tyrosine kinase could be inactivated by specific small-molecule inhibitors[55]. Currently, targeted agents such as crizotinib, alectinib or ceritinib are clearly superior to chemotherapy in patients with *ALK*-rearranged NSCLC[56–59]. Furthermore, crizotinib has been also approved for patients harboring rearrangements that involve the ROS Proto-Oncogene 1 (*ROS1*) tyrosine kinase, which is observed in 1% of the patients with advanced NSCLC[60].

Recently, new therapies that target the interaction between the immune system have entered the clinic. Of these, pembrolizumab (a monoclonal antibody targeting the Programmed Cell Death Protein 1, also referred to as PD-1) has been approved for the first-line treatment of patients whose tumors express Programmed Death Ligand 1 (PD-L1) in ≥50% of cancer cells

cells (see below), which represents approximately 20-30% of the patients diagnosed with advanced NSCLC without *EGFR* or *ALK* rearrangements.

Currently, although a subset of NSCLC patients may benefit from targeted treatment, most of them are still only amenable to chemotherapy. Notably, although many other molecularly-defined subgroups of NSCLC have been identified in recent genomic profiling studies[61,62], no additional targeted therapy has yet reached our daily clinical practice for molecularly selected NSCLC patients.

Adequate patient selection criteria are crucial for identifying those patients that will be amenable to a given targeted therapy. Successful patient selection is commonly based on histologic or molecular traits that serve as predictive biomarkers of treatment benefit. In NSCLC, the role of MET and PD-L1 as biomarkers for patient selection is currently a matter of debate. The studies presented in this thesis focus on the analysis of MET and PD-L1 in NSCLC specimens. Thus, the next lines will be dedicated to the relevance of MET and PD-1/PD-L1 as targets for anti-cancer therapy and the current limitations of patient selection based on MET alterations and PD-L1 expression for their respective targeted therapies.

1.3. MET

1.3.1. Relevance of MET in cancer

The MET Proto-Oncogene (MET) is located at 7q31 and encodes a transmembrane receptor with tyrosine kinase activity[63]. In physiological conditions, MET is the natural receptor for hepatocyte growth factor (HGF), also called scatter factor[64,65]. The HGF/MET axis is essential for mammalian embryogenesis, participating in placental, liver and muscle development[65–67]. In adults, HGF/MET signaling plays an important role in the response to tissue damage and wound healing[68,69]. Upon ligand binding, active MET homodimers are formed, leading to the phosphorylation of the tyrosine kinase and substrate-binding domains[70]. This triggers the recruitment of several intracellular effector proteins such as GAB1 and GRB2[71] (Figure 2). MET activation further stimulates signaling through several downstream pathways implicated in cell growth, survival and migration[72].

MET was first identified as an oncogene in an osteosarcoma cell line and was later found to be implicated in tumor metastasis, hence its name[73,74]. The transforming potential of activated MET has been demonstrated in several experimental models, confirming that it can cause cancer in humans[75,76]. Signaling through MET is mainly mediated through the MAPK and PI3K-

AKT pathways (Figure 2)[72,77]. However, one important feature of MET activation is its potential crosstalk with other signaling pathways. Indeed, co-activation of MET with other tumor drivers such as IGFR, EGFR or HER2, has been described in different experimental cancer models[78–80]. Furthermore, increased transcription of *MET* can be observed after Wnt-pathway activation and MET signaling can activate angiogenesis[81,82].

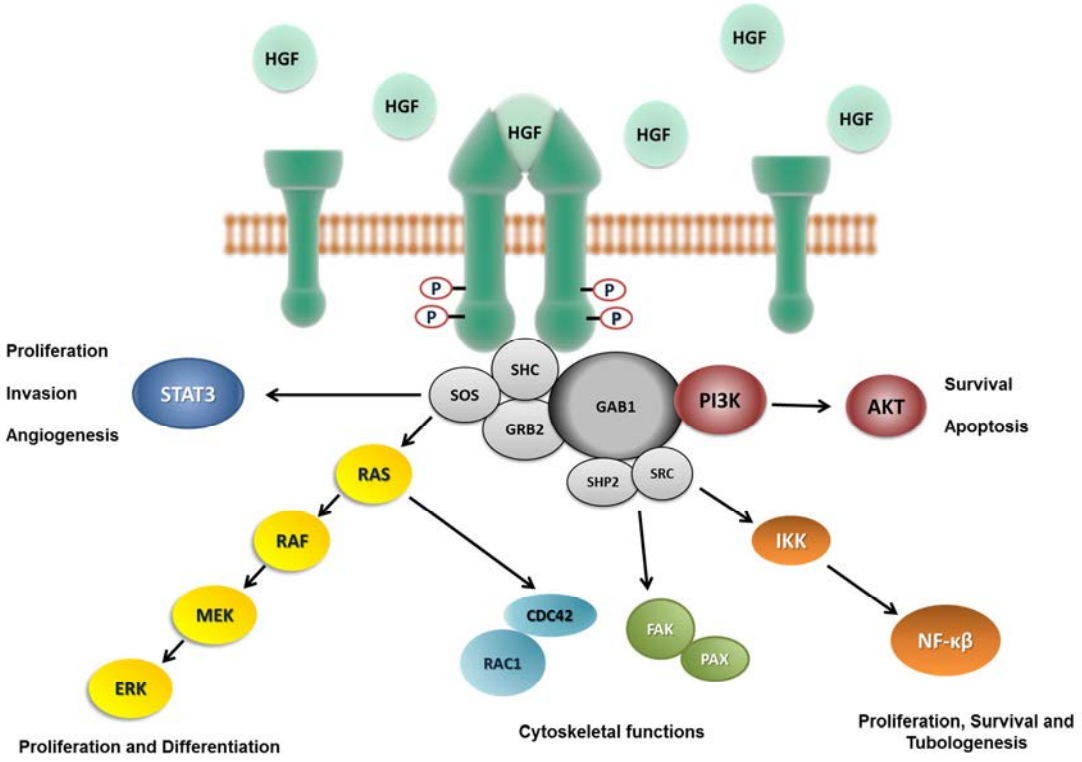


Figure 2. Signaling pathways after MET receptor activation. Green transmembrane molecules represent the MET receptor monomers. A MET dimer is formed after ligand (HGF) binding and this is followed by downstream activation of multiple oncogenic pathways.

Activating *MET* mutations have been described in sporadic and hereditary papillary renal cancers as well as in melanoma, lung and ovarian carcinomas[83,84]. Moreover, *MET* amplification can be found in gastric and colon[85–87]. Finally, *MET* amplification has been also detected in NSCLC cell lines, and knockdown of MET by specific shRNAs can lead to growth inhibition and apoptosis in *MET*-amplified NSCLC cells, providing a preclinical rationale for MET-directed therapies in lung cancer[88].

1.3.2. MET alterations in NSCLC

The crucial role of MET in human cancer development and progression has led to active investigation of this receptor as a potential therapeutic target. Expression of HGF and MET in NSCLC was first reported more than 20 years ago, and several clinical and preclinical studies

have highlighted its potential relevance in NSCLC biology. Initial studies that analyzed MET protein overexpression in NSCLC samples reported it as an adverse prognostic factor[89–92]. Later studies have claimed a similar prognostic effect for *MET* gene amplification[93–98], and this hypothesis is sustained by two recent meta-analyses[99,100]. A critical role for *MET* amplification as a putative resistance mechanism to erlotinib and gefitinib in *EGFR*-mutant NSCLC was strongly suggested by two independent preclinical studies, and further examination of clinical samples identified *MET* amplification in up to 22% of TKI resistant cases[87,101].

Following these results, many investigators set out to validate the clinical relevance of MET alterations in NSCLC (Table 1). However, these efforts have been hampered by the lack of consensus in defining MET "positive" tumors. Regarding *MET* gene status, Cappuzzo et al. defined tumors as MET-positive when they harbored a mean *MET* copy number count of ≥5, since this was the optimal cut-off to define a subset of patients with worse overall survival (OS) in a retrospective study of 431 surgically resected NSCLC specimens[97]. Other authors (Table 1) followed the criteria for defining *HER2*-positive breast cancer[102] or adapted the University of Colorado Cancer Center (UCCC) criteria originally designed to assess *EGFR* gene amplifications[103] (see Tables 1 and 2). Briefly, these two criteria include specimens with true *MET* gene amplification (most frequently defined as MET/CEP7 ratio of ≥2 or tight *MET* gene clusters) as well as specimens with varying degrees of *MET* copy number gains, including cases with chromosome 7 polysomy. Finally, some authors opted for establishing internally validated or arbitrary cut-offs to define *MET* gene activation. Overall, MET positivity based on genetic criteria has been reported to be as high as 38.9% (Table 1)[104]. However, recent studies that have analyzed the rate of "true" *MET* amplifications (i.e. excluding polysomy) have reported ranges of 0.4-8.2%[104–106].

Attempts have also been made to define MET positivity using MET protein levels determined by immunohistochemistry (IHC). Most of the studies have employed semiquantitative approaches (H-scores) that combine intensity and extent of MET protein staining. Using this method, they have either defined arbitrary cut-offs for positivity or have commonly employed the median H-scores of their cohorts as positivity thresholds. Many investigators, however, have adapted the criterion defined in one of the early trials with MET inhibitor onartuzumab (see below). By this criterion, specimens showing ≥ 50% strong or moderate MET staining are considered MET IHC positive (Table 1). Of note, although many studies report a statistically significant correlation between MET IHC and FISH positivity (Supplementary Table 1), the actual overlap of the two techniques is remarkably low. As shown in Table 2, a high proportion of IHC-defined MET positive cases do not harbor *MET* gene alterations, while a non-negligible

proportion of MET FISH positive cases does not show MET protein overexpression. As expected, the rate of concordance varies according to the different evaluation criteria for each technique.

Therefore, although MET activation appears to define a poor prognostic subset of NSCLC patients in most cases, the exact criteria to define tumor MET-dependency using gene copy number or protein expression have not yet been established. This explains the differences in prevalence of MET alterations and may partly explain the lack of consistent correlations between clinico-pathological variables and MET alterations (Supplementary Table 1). Finally, in recent genomic profiling studies of clinical NSCLC specimens, mutations in exon 14 of the *MET* gene have been detected[107–110]. These mutations impair MET receptor degradation by modifying the ubiquitin ligase target segment of the receptor, leading to sustained MET signaling. *MET* exon 14 mutations have been reported in 3% of lung adenocarcinomas and 2.3% of non-adenocarcinoma cases and can occur concomitantly with *MET* gene amplification[107,110].

1.3.3. Selection criteria for MET targeted therapy in NSCLC

The most promising results thus far with MET inhibitors in NSCLC have been obtained with crizotinib, a currently approved agent for patients with *ALK* or *ROS1* rearranged NSCLC that is also a potent inhibitor of the MET tyrosine kinase[111]. Remarkably, however, crizotinib efficacy appears to be confined to stringently selected patients. Preliminary results of a phase I/II clinical trial conducted by Camidge and collaborators showed higher response rates in patients with high-level *MET* amplification (defined by a MET/CEP7 ratio of ≥5; response rate 50%; n=6) compared with patients without this feature (response rate 14.3%; n=7)[112]. Furthermore, crizotinib is also effective in the small subset of NSCLC patients with *MET* exon 14 skipping mutations, showing durable responses[110,113,114]. Although there might exist an overlap between *MET* mutations and amplification, crizotinib has also shown efficacy in patients with high-level *MET* amplification without evidence of mutations[115,116].

Before crizotinib, the only drugs that have ever been studied in phase III trials failed to reach the clinic. The first of such drugs was the small molecule tivantinib. Tivantinib showed promising efficacy in solid tumors in combination with erlotinib[117]. Further development in NSCLC suggested that its efficacy was greater in patients with nonsquamous histology than in patients with squamous cell carcinomas[118,119]. The phase III trial (MARQUEE) involved over one thousand nonsquamous NSCLC patients that had progressed after first-line chemotherapy and were randomized to tivantinib/erlotinib or placebo/erlotinib, but failed to prove an overall

survival benefit for the patients treated in the experimental arm[120]. Most remarkably, a recent study in NSCLC cell lines with and without *MET* amplification found that the anticancer effect of tivantinib was independent of MET signaling and that tivantinib was unable to abrogate MET downstream signaling, as opposed to other known MET inhibitors[121].

Onartuzumab, a monovalent antibody directed against the extracellular SEMA domain of the MET receptor, showed promising activity in a phase I trial including heavily pre-treated NSCLC patients[122]. The phase II trial conducted in previously treated NSCLC patients reported a benefit when onartuzumab was added to erlotinib, although only in the subset of patients considered MET positive by IHC (Metmab criterion: ≥ 50% strong or moderate MET positivity in tumor cells)[123]. The following phase III trial (MetLung) included only Metmab positive patients that had progressed after first-line chemotherapy. Similarly to what happened in the MARQUEE trial with tivantinib, onartuzumab+erlotinib failed to prove a benefit in terms of OS compared with placebo+erlotinib[124].

The failure of multiple trials of MET-directed therapies and the preliminary results observed with crizotinib highlight the relevance of adequate patient selection for targeted therapy. Probably, the population that presents high response rates with crizotinib includes patients whose tumors are truly driven by MET activation and, presumably, the selection criteria of the onartuzumab and tivantinib trials failed to identify this population. Moreover, the preclinical studies that had suggested the importance of MET in NSCLC were conducted in *EGFR*-mutant cell lines and patients with *EGFR*-mutant NSCLC and acquired resistance to EGFR TKI. However, both onartuzumab and tivantinib were tested in molecularly unselected populations. Of note, a trial with tivantinib conducted in an Asian population (ATTENTION) included only *EGFR* wild-type patients. Furthermore, the relevant MET alteration identified in preclinical studies was gene amplification. Remarkably, many studies have shown that MET amplification does not always correlate with MET IHC (Table 2). However, onartuzumab was essayed in MET "positive" population defined by IHC criteria that had only been internally validated[125]. Finally, according to the experience with crizotinib, the most promising biomarker for MET inhibition efficacy is high-level *MET* amplification as defined by Camidge et al[112], and the results of ongoing clinical trials are eagerly expected. Hopefully, future studies will reveal the true role of *MET* mutations and *MET* amplifications as druggable targets in NSCLC.

1.4. PD-L1

1.4.1. Immune tolerance and the PD-1/PD-L1 axis in cancer

One of the main hallmarks of cancer is the avoidance of immune destruction. Thus, while cancer cells might be initially targeted and destroyed by the host's immune system, they eventually escape immune recognition during cancer evolution[126,127]. One of the mechanisms by which cancer cells achieve this is through the induction of immune tolerance. Immune tolerance is mediated by a variety of mechanisms that involve immune cells, membrane molecules and soluble cytokines and chemokines. Some of these mechanisms consist of receptor-ligand interactions (also called immune checkpoints) that ultimately down-regulate immune function[128]. One of the many immune checkpoints that mediate tolerance is the pathway of the PD-1 receptor and its ligand PD-L1. It is known that this pathway can be co-opted by cancer cells to escape immune surveillance, suggesting a rationale for targeted therapies[129,130]. Indeed, targeted blockade of the interaction between PD-1 and its ligands has yielded significant results in solid tumors, changing the therapeutic landscape of many tumor types (see Figure 3 on the following page)[131–135]. However, like other targeted therapies, it has been faced with the challenge of accurately identifying its target population.

PD-1 is usually expressed in activated lymphocytes, while its ligand PD-L1 is commonly expressed in macrophages. However, PD-L1 expression can be induced in other cell types such as epithelial or endothelial cells by cytokine-mediated signaling[130,136]. PD-1 has another ligand, PD-L2, which is normally expressed in macrophages and dendritic cells. However, while PD-L1 is known to play a role in peripheral immune tolerance, the immune modulatory role of PD-L2 is less well characterized[137]. Physiologically, the PD-1/PD-L1 axis acts in the late phase of the immune response, modulating T-cell function and limiting autoimmunity. Following antigen recognition, active T-cells up-regulate PD-1. Then, as the immune response evolves, inflammatory cytokines can induce PD-L1 expression in surrounding tissues to avoid collateral damage. Remarkably, however, this interaction can also favor the expression of both PD-L1 and PD-L2 in tumor cells[129].

1.4.2. PD-L1 staining as a selection criterion for PD-1/PD-L1 inhibitors in NSCLC

Initial trials with PD-1/PD-L1 inhibitors in solid tumors reported durable responses patients with advanced melanoma, renal cell carcinoma and NSCLC[131,132]. However, responses were confined to a small proportion of patients within each cancer type, while some tumor types presented no responses. Soon, efforts were focused on identifying predictive biomarkers of anti-PD-1/PD-L1 efficacy, a research that is still ongoing and has been faced with many hurdles.

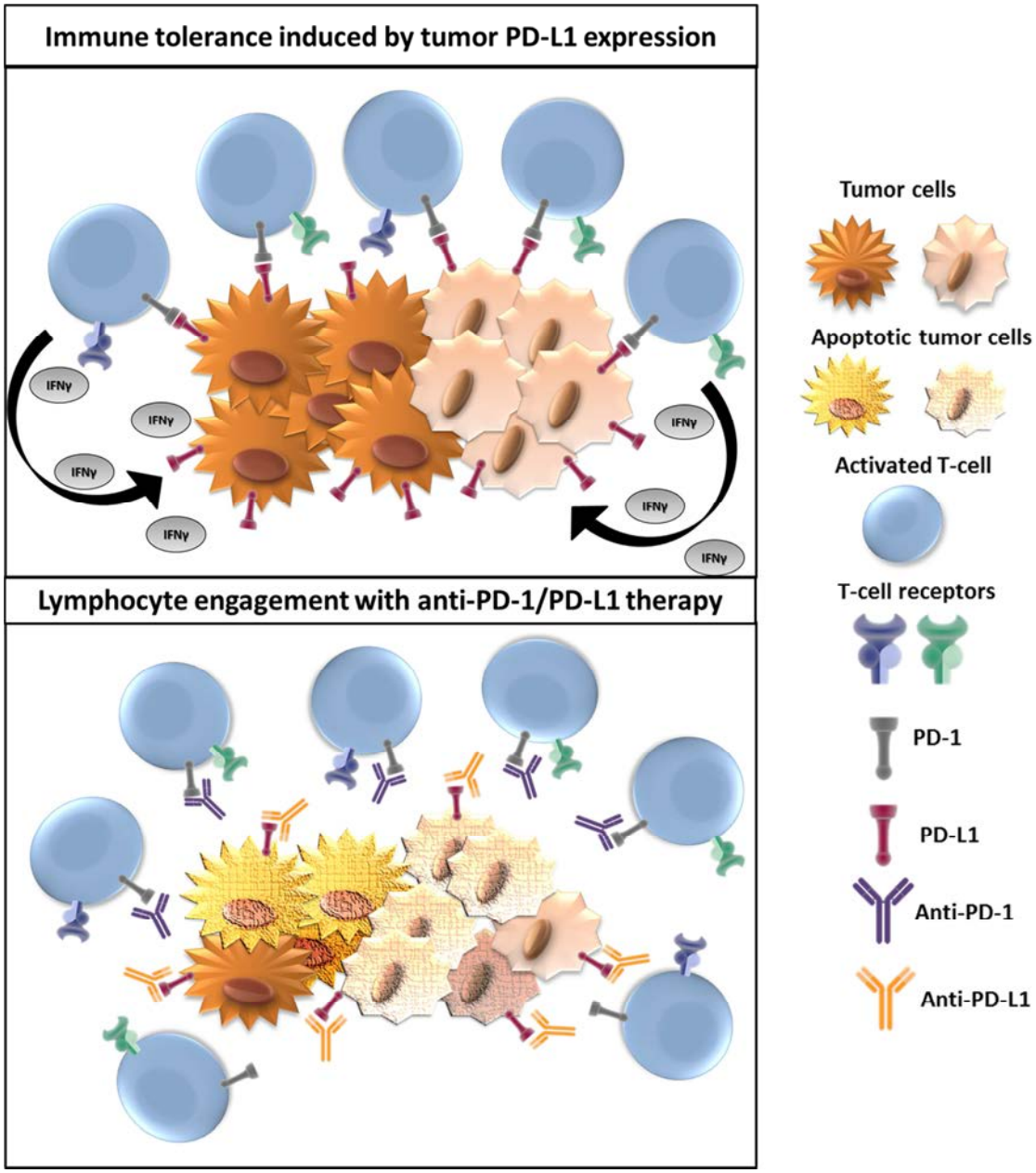


Figure 4. Immune destruction engaged by PD-1/PD-L1 inhibition. Schematic illustration of the mechanism of action of immune checkpoint inhibitors directed against PD-L1 and PD-L1. *IFN-γ, interferon gamma*

Early small cohort studies focused on the expression of several markers within the tumor parenchyma and the tumor microenvironment, suggesting a predictive role for activated CD8+, PD-1 and PD-L1 in pretreatment tumor specimens[138,139]. PD-L1 immunohistochemical staining emerged as the most promising biomarker in the early phase I with nivolumab (anti-PD-1) and atezolizumab (anti-PD-L1)[140,141]. Thereafter, PD-L1 expression became the commonly analyzed biomarker in anti-PD-1/PD-L1 trials. However, this biomarker has proven far from perfect, raising controversial issues regarding patient selection for cancer immunotherapy.

The initial success of immune checkpoint inhibitors targeting the PD-1/PD-L1 axis prompted the almost simultaneous development of multiple such drugs for many cancer types[132,140]. In NSCLC, two PD-1 inhibitors (nivolumab and pembrolizumab) and one PD-L1 inhibitor (atezolizumab) are already available, and two further anti-PD-L1 agents (durvalumab and avelumab) are reaching final stages of clinical development. In most of the trials with these agents, tumor PD-L1 immunohistochemical expression has been assessed and, in some studies, PD-L1 expression was required to enter the clinical trial. Of note, however, each drug has been developed alongside specific diagnostic assays, each involving a different antibody clone and reading platform as well as specific cut-offs for PD-L1 positivity. Furthermore, only the SP142 immunohistochemical assay (employed in trials with atezolizumab) includes the PD-L1 staining of immune cells as part of the evaluation criteria, while the others only take tumor PD-L1 positivity into account (see Table below).

Diagnostic assays and PD-L1 positivity criteria according to different anti-PD-1/PD-L1 agents

Drug	Target	Antibody	Platform	Compartment	Cut-off
Nivolumab	PD-1	28-8	Link 48 Autostainer	Tumor	≥1%
Pembrolizumab	PD-1	22C3	Link 48 Autostainer	Tumor	≥50%
Atezolizumab	PD-L1	SP142	BenchMark ULTRA	Tumor and immune cells	≥1%
Durvalumab	PD-L1	SP263	BenchMark	Tumor	≥25%
Avelumab	PD-L1	73-10	Dako assay	Tumor	≥1%

Interestingly, although high PD-L1 positivity rates (irrespective of cut-off or cellular compartment) seem to enrich the population for a higher proportion of responders to anti-PD-1/PD-L1 therapies, absence of PD-L1 expression does not exclude patient benefit from such agents (see Table 3A-D). Moreover, these important methodological differences in drug/biomarker development have led to a lack of uniformity in trial results and interpretation of the clinical efficacy of each one of these drugs. This, in turn, can lead to differences in drug approval by regulatory agencies and thus affecting drug availability for patients and clinicians. For example, pembrolizumab and nivolumab, two drugs that share the same target, have strikingly different approval statuses. Pembrolizumab is approved for untreated NSCLC patients whose tumors show ≥50% of PD-L1 positive tumor cells (as determined by the 22C3 companion diagnostic assay) due to a clear benefit over chemotherapy in a phase III trial[142]. On the other hand, nivolumab failed to prove increased efficacy compared with chemotherapy in the first-line setting using a 5% PD-L1 positivity cut-off[143]. In contrast, both agents are available

in the second-line setting, pembrolizumab in patients with ≥1% PD-L1 positive tumor cells and nivolumab in all patients, irrespective of PD-L1 staining[144–146].

Thus, increasing evidence suggests that PD-L1 staining may be inadequate for selecting patients that will benefit from PD-1/PD-L1 targeting therapies[147]. However, current available data from clinical trials forces oncologists and pathologists to ensure the availability of multiple PD-L1 diagnostic assays in order to be able to prescribe anti-PD-L1/PD-1 drugs or to choose a specific PD-L1 antibody clone based on low-evidence studies. This represents a challenge especially for pathologists, since they have to be familiar with each assay and their respective immunohistochemical evaluation criteria. Furthermore, the necessity for specific reading platforms for each IHC assay raises important feasibility and sustainability questions.

1.5. Intratumor heterogeneity and biomarker assessment in NSCLC

As illustrated throughout the previous sections of this text, biomarker assessment is crucial for targeted therapy development and success. However, despite important research efforts focused on biomarker discovery, less than 1% of biomarkers reach the clinic. There are numerous possible causes for biomarker failure, a comprehensive review of which is beyond the scope of this text. Instead, we will focus on the intratumor heterogeneous expression of biomarkers, a phenomenon that has been hitherto largely ignored in clinical trials and biomarker validation studies.

The existence of intratumor heterogeneity (ITH) was already acknowledged in the midst of the 20th century, and the theory of cancer clonal evolution was first postulated forty years ago[148–150]. However, it has not been until recently, with the advent of high throughput genomic analysis and sophisticated analytical methods, that ITH has reached mainstream cancer research[151]. Today, by performing next-generation sequencing (NGS) of spatially separated regions of primary tumors and their corresponding metastases, the complex genomic landscape of different cancer types is being unveiled. Thus, by quantifying the proportion of clonal (present in all the cells of a tumor) and subclonal (present in less than 100% of tumor cells) genomic alterations, the extent of ITH and the chronological evolution of different tumors can be inferred. With this information, complex phylogenetic trees can be constructed, in which clonal alterations are mapped to the trunk, whereas subclonal alterations are located on the different branches[151–155].

In NSCLC, two retrospective studies published in 2014, suggested that ITH and branched evolution are a common phenomenon in NSCLC[156,157]. The first study[156], led by de Bruin et al, performed multiregion whole-exome and whole-genome sequencing on 25 spatially separated areas of seven surgically-treated NSCLC patients. The median fraction of heterogeneous mutations between different regions was 30% (range 4 to 63%). Notably, the probability of missing a potential driver alteration by analyzing only one tumor region ranged from 42 to 83%. However, driver mutations were more frequently located at the trunk region of the phylogenetic tree, suggesting they were mostly ubiquitous and occurred early during carcinogensis. In contrast, copy number alterations, which also contributed to ITH, appeared to occur at later cancer evolutionary stages[156].

The second study[157], led by Zhang et al, performed whole-exome sequencing on 48 different regions of 11 patients with localized NSCLC. They found that approximately 76% of mutations were shared among the different tumor regions of a same tumor. Furthermore, of the 14 non-synonymous mutations in known cancer genes, 13 (92.8%) were mapped to the trunk of the evolutionary tree. Moreover, they did not find significant inter-regional differences regarding large-scale chromosomal aberrations or cancer gene copy number alterations. The authors concluded that known oncogenic events occurred early during NSCLC evolution and thus were shared among different subclones. Remarkably, the three patients that presented disease relapse in this study (median follow-up of 21 months) had a significantly higher mean fraction of subclonal mutations compared with patients without a relapse (40% vs. 17%, respectively; $p=0.006$)[157].

In summary, both studies found a similar overall rate (30% and 24%) of subclonal mutations, proving the existence of genomic ITH and branched clonal evolution in NSCLC. Furthermore, they corroborated the existence of a high degree of genomic instability (i.e. high mutational load and a high prevalence of chromosomal aberrations) in NSCLC. Remarkably, both studies analyzed only thoracic-confined disease, suggesting that ITH and genomic instability are already present when cancer is first detected. Finally, both studies reported evidence of subclonal populations within the same regions, highlighting a potential impact of genomic ITH on biomarker assessment using single biopsies.

The TRACERx (Tracking Lung Cancer Evolution through Therapy; NCT01888601) study is expected to provide an accurate estimate of the true extent of ITH in NSCLC. TRACERx is a large, prospective study that plans to include over 800 patients with the objective of performing an in-depth analysis of ITH and its longitudinal variation, employing multiregion

NGS of surgical NSCLC specimens followed by a systematic patient follow-up regimen[158]. Recently, the analysis of the first 100 patients included in TRACERx was published[159]. Overall, the authors found a median of 30% (range 0.5 to 93%) subclonal somatic mutations and a median of 48% (range 0.3 to 88%) subclonal copy number alterations. Moreover, patients with above median (>48%) subclonal copy number alterations harbored a significantly higher risk of relapse in the multivariate analysis (hazard ratio, 3.70; 95% CI, 1.29 to 10.65; p = 0.01), while no such prognostic role was found for subclonal somatic mutations. Interestingly, 76% of subclonal alterations would be misclassified as clonal if only one tumor area were studied, and the analysis of single tumor areas identified significantly fewer driver alterations compared with multi-region analysis (p=0.004).

These results underscore the need to address ITH in NSCLC. In our daily clinical practice, most of NSCLC patients are diagnosed by means of small tissue samples or cytology specimens, upon which biomarker studies are performed. Moreover, clinicians cannot decide which tumor area is preferentially analyzed, since this is usually conditioned by tumor localization, patient characteristics and sampling technique. However, multi-region tumor biopsy strategies are not feasible, since they may entail potential risks for our patients.

Given the existence of genomic ITH in NSCLC, it is plausible to hypothesize that there is also ITH in biomarker expression. However, in contrast to genomic ITH, heterogeneous expression of biomarkers has been poorly characterized in NSCLC and is not addressed in clinical trials. In the two studies presented here, we report the existence of heterogeneous MET and PD-L1 expression. We suggest that this heterogeneity could, at least partially, explain biomarker failure of MET and PD-L1.

2. HYPOTHESIS AND OBJECTIVES

Hypothesis:

We hypothesized that the expression of MET and PD-L1 may be heterogeneous and could therefore impact biomarker-based patient classification in NSCLC.

Main objectives:

1. To study and quantify the heterogeneity of MET and PD-L1 in surgical NSCLC samples
2. To assess the impact of MET and PD-L1 heterogeneity on biomarker-based patient classification

Secondary objectives:

a) To study the correlation between biomarker alterations at the protein level (IHC) and the gene level (FISH)
b) To identify potential correlations of biomarker expression and heterogeneity with clinical features
c) To study the correlation between biomarker status and other histopathological and molecular variables

3. MATERIALS AND METHODS

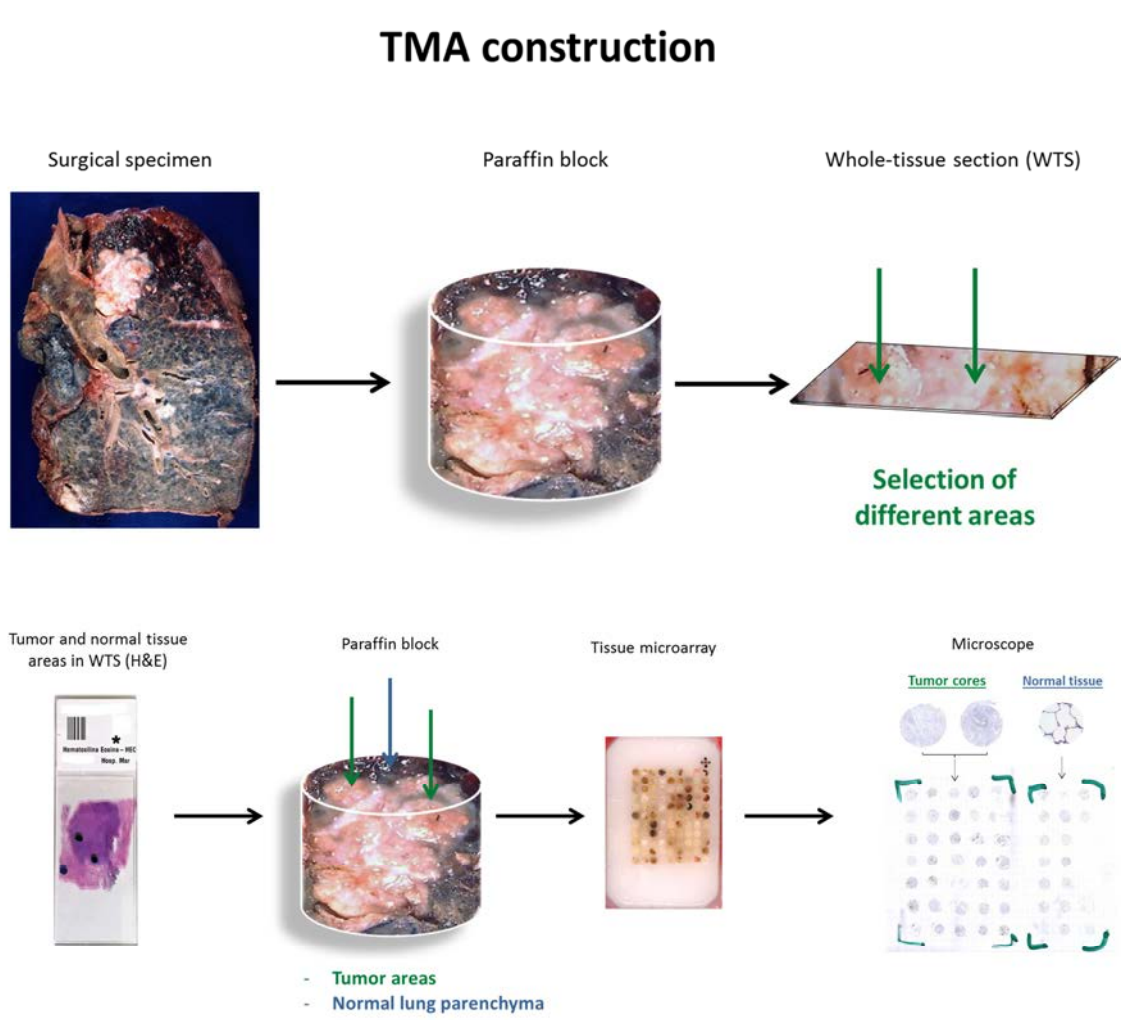
Population of the studies:

Both studies presented here included patients diagnosed of NSCLC at Hospital del Mar (Barcelona). Patients were selected based on tissue availability. For the first study, in which we assessed heterogeneity of MET, only patients with nonsquamous histology were admitted. In contrast, for the second study, which looked at PD-L1 expression, a subset of squamous cell NSCLC specimens was also included.

Clinical, pathology and molecular data were retrieved from digitalized medical records. Clinical data included sex, age, smoking history, disease stage (7^{th} edition of the International Association for the Study of Lung Cancer (IASCL) / Union Internationale Contre le Cancer (UICC) classification[6]), date of diagnosis (for surgical specimens, the date of surgery was taken into account), date of relapse, date of last-follow-up, and status at last follow-up (alive/deceased). Pathology and molecular data included predominant growth pattern (only for adenocarcinomas), tumor grade, *EGFR* mutations, *KRAS* mutations, and *ALK* rearrangements.

Tissue microarray construction:

Incorporation of tumor specimens into tissue microarrays was performed according to Kononen et al[160] as illustrated in the schema (next page). First, Hematoxylin-Eosin (H&E) stained-sections from formalin-fixed paraffin-embedded tumor blocks were reviewed by a senior pathologist (Dr. Lara Pijuan). Tumor areas were selected and 1mm-diameter cores extracted and incorporated into a tissue microarray. Each TMA contained two cores from the same patient, each belonging to a different tumor area. For adenocarcinoma specimens, areas with different growth patterns or distinct morphological traits were selected. For squamous cell carcinoma specimens, spatially separated areas were chosen. The first study (MET heterogeneity) included four cores per patient, while the second study (PD-L1 heterogeneity) included only two cores per patient. Cores were labeled A,B,C,D in the first study and A,B in the second study. Techniques for biomarker analysis and scoring methods were performed according to specific protocols and criteria, which are detailed in the methods section of each one of the articles (see Results). Additional images illustrating MET and PD-L1 expression and heterogeneity are included in the supplementary material of the articles and in APPENDIX II.



Shematic illustration of TMA construction. Images from the surgical specimen, TMA block and normal parenchyma core were retrieved from Google Images under a Creative Commons License. Image of the whole tissue section slide (with selected areas for core extraction) and the final microscopic view of the TMA and the tumor cores correspond to examples of cases included in our studies.

Statistical analysis:

Statistical associations were studied between patient biomarker classification and other clinical variables. For these analyses in the TMA populations of the studies, the highest scoring core per patient was taken into account (except in the first study for MET immunohistochemistry, in which a combined score of the four TMAs was performed). Secondly, to study the association of biomarker status and other pathology and molecular variables, each TMA core was counted as an individual specimen.

Heterogeneity studies were performed analyzing the rate of total discordant core pairs divided by the total of core pairs analyzed, excluding those pairs in which one or both of the cores were considered not evaluable. Additionally, disagreement of biomarker scoring between cores was studied using the kappa agreement index for categorical variables and the intraclass correlation coefficient for continuous variables.

Association between categorical variables was studied using Chi-square and Fisher's exact test. Association between continuous variables was studies using two-sample T-tests and Kruskall-Wallis test as deemed pertinent.

Survival differences were evaluated using the Cox regression test and plotted using Kaplan-Meier curves. Statistical analysis was carried out with SPSS version 13.0 (SPSS Inc., Chicago, IL).

4. RESULTS

Article 1. MET expression and copy number heterogeneity in nonsquamous non-small cell lung cancer (nsNSCLC)

Authors: David Casadevall, Javier Gimeno, Sergi Clavé, Álvaro Taus, Lara Pijuan, Miriam Arumí, Marta Lorenzo, Silvia Menéndez, Israel Cañadas, Joan Albanell5 Sergio Serrano, Blanca Espinet, Marta Salido, and Edurne Arriola

MET expression and copy number heterogeneity in nonsquamous non-small cell lung cancer (nsNSCLC)

David Casadevall[1,6,*], Javier Gimeno[2,*], Sergi Clavé[3,4,*], Álvaro Taus[1], Lara Pijuan[2], Miriam Arumí[1], Marta Lorenzo[4], Silvia Menéndez[4], Israel Cañadas[4], Joan Albanell[1,4,5], Sergio Serrano[2], Blanca Espinet[3,4], Marta Salido[3,4], Edurne Arriola[1,4,#]

[1]Servei d'Oncologia Mèdica, Hospital del Mar, Barcelona, Spain

[2]Servei de Patologia, Hospital del Mar, Barcelona, Spain

[3]Laboratori de Citogenètica Molecular, Servei de Patologia, Hospital del Mar, Barcelona, Spain

[4]Cancer Research Program, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain

[5]Universitat Pompeu Fabra, Barcelona, Spain

[6]Universitat Autònoma de Barcelona, Bellaterra, Spain

[#]Cancer Sciences Unit, University of Southampton, Southampton, UK

[*]These authors have contributed equally to this work

Correspondence to:
David Casadevall, **e-mail:** dcasadevall@parcdesalutmar.cat

Keywords: *c-MET*, *immunohistochemistry*, *FISH*, *non-small-cell lung cancer*, *heterogeneity*

Received: *February 25, 2015* ***Accepted:*** *May 05, 2015* ***Published:*** *May 15, 2015*

ABSTRACT

Objective: We aimed to assess MET intratumoral heterogeneity and its potential impact on biomarker-based patient selection as well as potential surrogate biomarkers of MET activation.

Methods: Our study included 120 patients with non-squamous Non-small-cell Lung Cancer (nsNSCLC), of which 47 were incorporated in tissue microarrays (TMA). Four morphologically distinct tumor areas were selected to assess MET heterogeneity. MET positivity by immunohistochemistry (IHC) was defined as an above-median H-score and by +2/+3 staining intensity in >50% of tumor cells (Metmab criteria). *MET* FISH positivity was defined by *MET*/CEP7 ratio ≥ 2.0 and/or *MET* ≥ 5.0. MET staining pattern (cytoplasmic vs. membranous) and mesenchymal markers were investigated as surrogates of MET activation.

Results: Median MET H-score was 140 (range 0–400) and 47.8% of patients were MET positive by Metmab criteria. Eight cases (6.8%) were *MET* FISH positive and showed higher H-scores (p = 0.021). MET positivity by IHC changed in up to 40% of cases among different tumor areas, and *MET* amplification in 25–50%. Cytoplasmic MET staining and positivity for vimentin predicted poor survival (p = 0.042 and 0.047, respectively).

Conclusions: MET status is highly heterogeneous among different nsNSCLC tumor areas, hindering adequate patient selection for MET-targeted therapies. MET cytoplasmic staining and vimentin might represent surrogate markers for MET activation.

INTRODUCTION

Despite significant advances in diagnosis and treatment, lung cancer remains the leading cause of cancer death worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for up to 85% of lung cancers, of which 40% are adenocarcinomas [2]. During the last decade, considerable progress has been made in the knowledge of NSCLC biology. Several molecular alterations, such as mutations in the epidermal growth factor receptor (*EGFR*)

[3] or anaplastic lymphoma kinase (*ALK*) and ROS proto-oncogene 1 (*ROS1*) rearrangements [4]predict response to specific targeted therapies. These developments have greatly impacted on patients' outcome and quality of life [5–7].

MET was first identified in the late '80s, it is located on chromosome band 7q31 and encodes a heterodimeric transmembrane receptor with tyrosine kinase activity (RTK) [8, 9]. Activation of MET initiates a cascade of cellular signaling processes that ultimately lead to proliferation, reduced apoptosis, epithelial to mesenchymal transition (EMT) and an increased invasiveness and metastatic potential [10, 11]. MET pathway activation has been explained by different mechanisms such as genetic point mutations, gene amplification, post-translational activation, as well as in a ligand-dependent manner [12, 13].

The presence of MET protein overexpression and *MET* gene amplification in NSCLC are globally considered as adverse prognostic factors [14–17]. Consequently, many efforts have been made to develop MET-targeted agents [18, 19]. Clinical benefit was initially reported in patients with high serum levels of circulating HGF [20]or whose tumors harbored *MET* gene amplification [21]. In the MARQUEE [22] and the MetLung trials [23], patients were selected based on non-squamous histology and on MET immunohistochemical expression, respectively. Both trials failed to meet their primary endpoints, highlighting the need for predictive biomarkers for Met-directed treatment.

During the past few years, next-generation sequencing studies have revealed remarkable genetic and phenotypic differences among individual solid tumors [24] and also among different tumor areas and their metastases [25, 26]. This heterogeneity can interfere with biomarker-based treatment decisions, particularly when these are made based on material from small tumor biopsies.

Finally, a recent report in patients with gastric adenocarcinoma has suggested that MET staining pattern can predict *MET* gene amplification [27]. Moreover, in previous experiences with SCLC patients, we have observed that total MET protein expression does not always translate pathway activation and that signaling through MET can trigger EMT [28]. Thus, we hypothesized that the presence of a mesenchymal phenotype could translate MET pathway activation.

The primary aim of this study was to evaluate the potential impact of intra-tumor heterogeneity on MET evaluation and classification using different techniques and criteria. Furthermore, we sought to assess the correlation of MET status with other pathological and molecular characteristics. Finally and with exploratory purposes, we investigated potential surrogate markers of MET activity, such as MET staining pattern and the presence of mesenchymal markers by immunohistochemistry.

MATERIALS AND METHODS

Study population

Criteria for patient selection were non-squamous non-small cell lung carcinoma (nsNSCLC) histology and availability of tissue for the studies. A total of 124 tumor specimens from 120 patients diagnosed of nsNSCLC at our institution between 2009 and 2013 were included. Four of the 120 patients presented two different tumors, thus providing one extra specimen each. Material was available either from surgical resections, core-needle biopsies or cytological cell-blocks. Clinical data were extracted from medical records and included age, sex, smoking history, tumor disease stage and clinical follow-up information.

Tissue microarray construction

Based on tissue availability, 47 of the patients were selected to construct tissue microarrays (TMAs) as outlined by Kononen *et al*. [29]. First, original Hematoxylin–Eosin (H&E) stained-sections were reviewed from each patient to identify different malignant areas and benign lung tissue. A total of six tissue cores with a 2 mm of diameter were obtained from each patient, four of them containing different histological areas of the carcinoma (named A, B, C and D) and two of them containing benign lung parenchyma. Two of the 47 patients presented two different tumors, thus providing eight tumor cores each. This lead to a final number of 196 tumor cores divided into six TMAs.

Fluorescence *in situ* hybridization

MET fluorescence *in situ* hybridization (FISH) evaluation was performed on unstained formalin-fixed and paraffin-embedded(FFPE) tissue sections from the whole tumor and the TMA samples, as previously described [30], using a *MET*/CEP7 probe cocktail (#06N05-020, Abbott Molecular Inc, Des Plaines, IL) according to manufacturer's instructions. A minimum of fifty non-overlapping cells with hybridization signals were examined for each case with a BX51 fluorescence microscope (Olympus, Tokyo, Japan) and using the Cytovysion software (Applied Imaging, Grand Rapids MI). Tumors with *MET*/CEP7 ratio $\geq$ 2.0 (named "truly amplified") and/or *MET* $\geq$ 5.0 copies (named "high polysomy") were considered *MET* FISH positive [15, 31]. *MET* gains -defined as a mean copy number $\geq$ 2.5 copies in at least 10% of analysed nuclei- were also recorded.

Immunohistochemical assays

MET immunohistochemistry (IHC) evaluation was performed using anti-total c-MET (SP44) Rabbit Monoclonal as a primary antibody (#7904430, Ventana

Medical Systems, Tucson, AZ) and revealed using an Anti-RbOmniMap DAB Detection Kit (#760149, Ventana Medical Systems). The staining was carried out according to the manufacturer's protocol on a Discovery XT platform (Ventana Medical Systems). The primary antibody was incubated for 60 minutes. IHC staining was evaluated by one pathologist using two different methods. The first one was an H-score, as initially described to evaluate EGFR expression [32]. Briefly, this score ranges from 0 to 400 and results from the combination of the staining intensity (0–4) and the percentage of positive tumoral cells (0–100%) in each sample. Tumor samples were considered positive if their H-score was above median. The second one was the method described by Spigel and collaborators [33], which divides tumors into two different categories: MET high for cases presenting strong MET staining (+2 or +3) in more than 50% of tumoral cells and MET low for cases not fulfilling the former criteria. Met staining pattern, i.e. predominantly membranous vs. cytoplasmic, was assessed as described elsewhere [27].

E-cadherin and vimentin as EMT immunohistochemical markers were evaluated semiquantitatively [34]. Anti-human E-cadherin (NCH-38) mouse monoclonal primary antibody (#IR059, Dako, Carpinteria, CA) and anti-Vimentin (V9) mouse monoclonal primary antibody (#IR630, Dako) were evaluated. Both were revealed using the EnVision Flex visualization system (#K8010, Dako) and carried out according to the manufacturer's protocol using DakoAutostainer Plus. E-cadherin expression was evaluated as positive or "normal" when more than 50% of tumoral cells showed either membranous or cytoplasmatic staining. Vimentin expression was evaluated as positive or "acquired" when more than 5% of tumoral cells presented strong staining. For analysis purposes, samples showing positive E-cadherin expression were considered as having an epithelial phenotype, whereas samples showing acquired Vimentin staining were considered mesenchymal.

Statistical analysis

All 196 TMA cores were considered and analysed as individual cases to study the association between MET IHC and *MET* FISH with histopathological variables. These associations were analysed using Chi-square or two-sample *T*-tests as necessary. Heterogeneity between different cores (A, B, C and D) was assessed using Kappa agreement index for categorical variables (i.e. FISH categories) and intraclass correlation coefficient for continuous variables (i.e. MET H-score).

Survival analyses were only performed in those patients included in the TMAs as this was a more homogeneous population, being all surgically treated patients with early stage disease. Survival curves were obtained with the Kaplan-Meier method and significance of the differences in outcome was evaluated with the Cox regression test. Statistical analysis was carried out with SPSS version 13.0 (SPSS Inc., Chicago, IL). Data and statistical analysis reported are fully compliant with the REMARK guidelines [35].

RESULTS

Clinical and pathological characteristics of the study population

Patients' median age was 66 years, 69% were males and 52% were current smokers. Forty percent of the study population had stage I disease and 85% were adenocarcinomas (Table 1). Most of the samples showed moderate or poor histological differentiation (Grades 2–3). The predominant histological patterns in adenocarcinomas were acinar or solid with mucin production, whereas lepidic and micropapillary patterns were less common.

Mutational data was available for more than 90% of the cases. *KRAS* and *EGFR* mutations were found in 21% and 12% of the samples, respectively, whereas 2% of the cases presented *ALK* rearrangements. Patients included in the TMA study had similar characteristics, but with a higher proportion of patients with stage I disease (60%) and *EGFR* mutated cases (21%).

MET FISH analysis

MET status by FISH was evaluable in 117 out of 124 tumors (94.4%). We found eight *MET* positive cases (6.8%; 8/117). Four of these cases exhibited a *MET*/CEP7 ratio ≥ 2 (truly amplified) and the remaining four had five or more copies of the *MET* gene (high polysomy). *MET* gain was identified in 73 cases (62.4%), being most of them polysomic for chromosome 7 ($n = 60$) (Table 2). *MET* gains were more prevalent in adenocarcinomas with a predominantly solid histological pattern ($p = 0.011$) (data not shown). Different FISH patterns are illustrated in Supplementary Figure 1.

MET IHC

MET IHC was assessable in 115 out of 124 tumors (92.7%). According to MetMab criteria, 55 cases (47.8%; 55/115) were classified as MET high, and 60 cases (52.2%; 60/115) as MET low (Table 2). Median H-score was 140 (range 0–400). According to H-score, 56 tumors were classified as positive (H-score > 140) and 59 as negative (H-score $\leq$ 140). Comparing both scoring methods, three cases were classified differently, one case with an H-score of 140 was classified as MET high and two cases with H-scores of 160 and 180, respectively, were classified as MET low. MET membranous staining was generally coarser than cytoplasmic staining (Supplementary Figure 2).

Table 1: Global study population and TMA patients' characteristics

	Global population[1] (*n* = 120)	TMA population[2] (*n* = 47)
Age (yr)		
Median	66	66
Range	41–92	41–80
Sex, *n* (%)		
Male	83 (69)	29 (62)
Female	37 (31)	18 (38)
Smoking status, *n* (%)		
Never smoker	20 (17)	12 (25)
Former smoker	37 (31)	14 (30)
Current smoker	63 (52)	21 (45)
Stage, *n* (%)		
I[3]	50 (40)	29 (60)
II	19 (15)	8 (16)
III	20 (17)	10 (20)
IV	35 (28)	2 (4)
Histology, *n* (%)		
Adenocarcinoma	106 (85)	44 (90)
NOS	18 (15)	5 (10)
Histological Grade[4], *n* (%)		
1	16 (20)	12 (30)
2	33 (42)	17 (42)
3	30 (38)	11 (28)
Not assessable	27	4
KRAS, n (%)		
Wild-type	90 (79)	38 (83)
Mutated	24 (21)	8 (17)
Not Assessable	10	3
EGFR, n (%)		
Wild-type	101 (88)	38 (79)
Mutated	14 (12)	10 (21)
Not assessable	9	1
ALK, n (%)		
Not rearranged	106 (98)	39 (95)
Rearranged	2 (2)	2 (5)
Not assessable	16	8

[1]Includes 4 patients who had two different tumors (*n* = 124 tumors).
[2]Includes 2 patients who had two different tumors (*n* = 49 tumors).
[3]Includes 4 stage 0 patients.
[4]Only *n* = 106 adenocarcinomas. *TMA,* Tissue microarray; *NOS*, Not otherwise specified.

Heterogeneity assessment

Heterogeneity studies were focused on the TMA population, in which 171 out of 196 cores (87.2%) were assessable for histology, 176 (89.8%) for grade, 184 (93.9%) for MET IHC and 180 (91.8%) for *MET* FISH. As expected, histological pattern and grade showed a highly heterogeneous distribution among different cores

Table 2: MET IHC and *MET* FISH status among biopsy (left) and TMA (right) specimens

	Global population[1] (*n* = 115)	TMA population (*n* = 49)
MET H-score		
Median	140	90
Range	0–400	0–400
Metmab score, *n* (%)		
MET high	55 (48)	17 (35)
MET low	60 (52)	32 (65)
	Global population[1] (*n* = 117)	**TMA population (*n* = 49)**
MET FISH negative		
MET disomic	36 (30.8)	11 (23)
MET gain	73 (62.4)	35 (71)
MET FISH positive[2]		
High polysomy	4 (3.4)	2 (4)
Truly Amplified	4 (3.4)	1 (2)

[1]For FISH analysis, the core with the highest gene copy number value was selected. For IHC, H-score and Metmab score was calculated using all 4 cores (see Materials and Methods).
[2]FISH positivity was defined as the average number of *MET copies* ≥5 or a *MET*/CEP7 ratio ≥2.
IHC, immunohistochemistry; *TMA,* Tissue microarray; *FISH,* fluorescence *in situ* hybridization.

(Kappa agreement index of 0.10 and 0.18, respectively, comparing A-B cores). When MET IHC status was analyzed considering the H-score as a continuous variable, intraclass correlation coefficient (ICC) was 0.47 between cores A and B. When all four cores (A to D) were included in the analysis, ICC was 0.57. When cases were classified using MetMab criteria and divided into MET high and Met low, comparison of core A with the remaining three cores (B to D) revealed differences in classification in approximately 20–40% of the cases (Figure 1).

Regarding *MET* FISH analysis, when evaluated as a categorical variable (*MET* disomic, *MET* gain, *MET* positive), Kappa agreement index between cores A and B was 0.35. Regarding *MET* gain as a continuous variable, ICC between the four cores was 0.58. Among the three *MET* FISH positive cases found in the TMA population, four out of the 12 cores represented were FISH negative. Moreover, none of the cases was considered positive in all four cores (Figure 2). Intra-tumor heterogeneity of MET by both IHC and FISH is illustrated in Figure 3.

Association between IHC and FISH

MET FISH positive cases had higher H-score values (p = 0.021) (Supplementary Figure 3). Among these, the four truly amplified cases had higher H-score values than those categorized as high polysomy 7, although these difference was not statistically significant (data not shown). However, no significant association was found between *MET* mean copy number and MET H-score considered as continuous variables (Supplementary Table 1). Applying the criteria recently proposed by Camidge *et al*. [36], only the four cases categorized as truly amplified would be considered *MET* positive tumors. Of these, one case had high-level *MET* amplification (*MET*/CEP7 ratio ≥ 5) with an H-score of 400 and MET high by MetMab criteria, whereas the remaining three cases had an intermediate-level of *MET* amplification (*MET*/CEP7 ratio ≥ 2.2- < 5), of which one was classified as MET high and the remaining two as MET low by IHC. Discordance between IHC and FISH is illustrated in Figure 4.

MET staining pattern and mesenchymal markers

MET staining pattern was assessable in 132 cores. Out of these, only 14 (10.6%; 14/132) corresponding to 11 patients showed a predominantly cytoplasmic staining. Heterogeneity of MET staining pattern among different tumor cores was also observed (Figure 5). Three patients had predominantly cytoplasmic MET in two cores. The remaining 8 patients showed cytoplasmic staining in only one of the four cores. No patient had cytoplasmic MET in all four cores and none of the cores with cytoplasmic MET was FISH positive (Supplementary Figure 4). Interestingly, predominant cytoplasmic MET staining correlated with lower MET H-scores (p = 0.003), whereas non-smoking was associated with a membranous staining pattern (p = 0.042) (Table 3).

		CORE B		CORE C		CORE D	
		High	Low	High	Low	High	Low
CORE A	MET high	13	9	14	9	11	12
	MET Low	4	17	2	23	6	15
Percent change		30.2% (13/43)		22.9% (11/48)		40.1% (18/44)	

Figure 1: Metmab status discordance among different tumor cores. Differences in MET IHC classification among different areas represented in each core. The highest variability was observed between cores A and D and the lowest between A and C.

	CASE 1		CASE 2		CASE 3	
	MET avg.	*MET*/CEP7	*MET* avg.	*MET*/CEP7	*MET* avg.	*MET*/CEP7
CORE A	5.7	1.14	5.3	1.16	9.1	2.57
CORE B	3.96	1.07	6.8	1.01	8.02	2.41
CORE C	6.02	1.00	3.02	1.06	4.18	1.12
CORE D	5.7	1.06	2.38	1.00	9.52	2.82

Figure 2: *MET* FISH discordance among different cores in FISH positive cases. Eight out of twelve cores are FISH positive. None of the cases shows FISH positivity in all four cores.

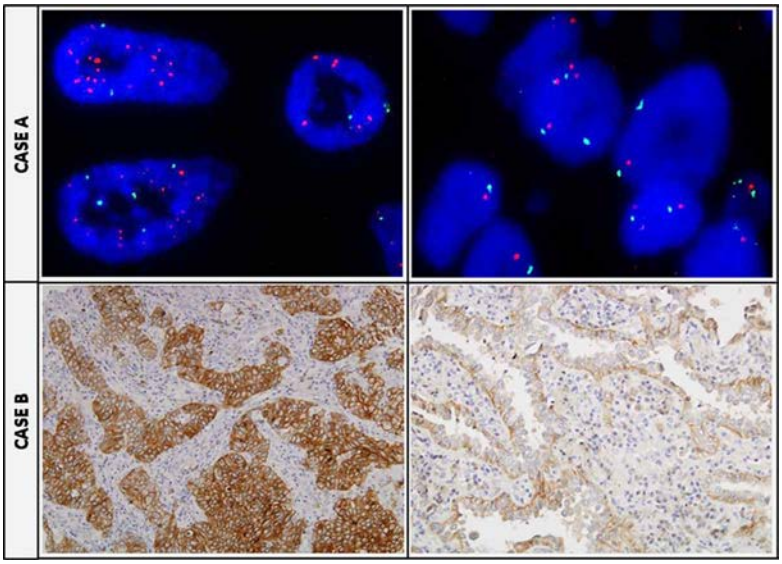


Figure 3: Tumor heterogeneity regarding MET status. CASE A. Two TMA cores of the same tumor sample with opposite FISH *MET* results: in the left a positive core showing a *MET/CEP7* ratio ≥2, and in the right a *MET* negative disomic case. **CASE B.** Two TMA cores of the same tumor sample with opposite MET IHC results: at the left a positive +4 area, and at the right a completely negative area of the same tumor.

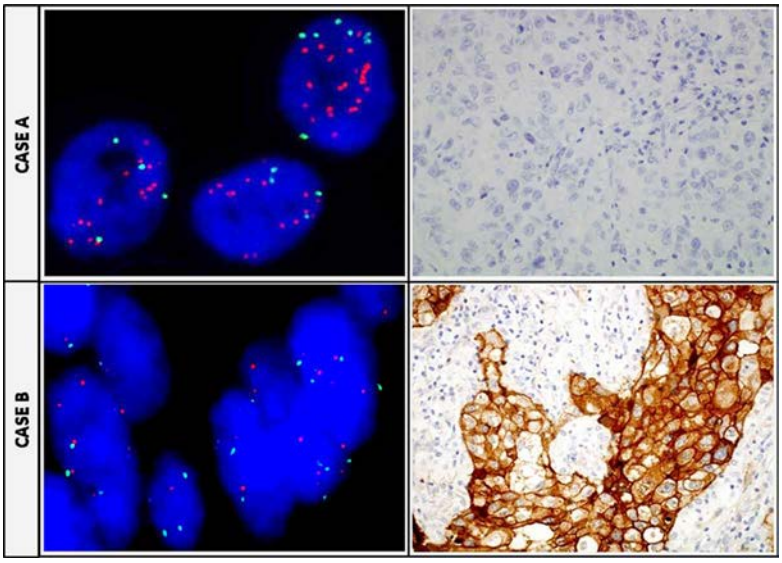


Figure 4: Discordance between FISH and IHC in individual tumors. CASE A. *MET* FISH positive case showing a *MET*/CEP7 ratio ≥2 (left) and, the same case assessed by IHC showing negative staining (right). **CASE B.** *MET* FISH negative sample (left) with a high positive score by IHC (strong +4 membranous predominant staining) in the same sample (right).

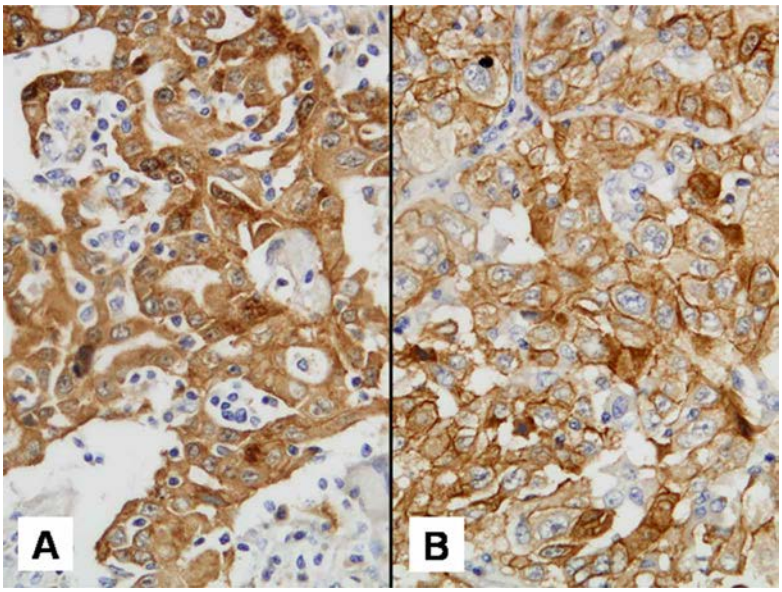


Figure 5: Met IHC staining pattern discordance. A. and **B.** show different tumor cores from the same patient. A: predominantly cytoplasmic staining and B: predominantly membranous staining.

Vimentin staining was assessable in 184 cores and E-cadherin in 181. A total of 19 cores corresponding to eight different patients (10.3%; 19/184) showed a mesenchymal phenotype (strong vimentin staining). All of these patients had a smoking history (five of them were current smokers and the remaining three were former smokers). Interestingly, the presence of a mesenchymal phenotype was associated with a predominantly cytoplasmic MET staining (p = 0.042). Also, tumors showing mesenchymal features had significantly lower H-scores (p = 0.027), whereas the opposite occurred for E-cadherin positive tumors, which had significantly higher H-scores (p = 0.003) (Table 3).

Survival analysis (TMA cohort)

Median follow-up time was 73.2 months and median survival time was not reached. One-, two- and three-year survival rates were 93.7%, 80.6% and 73.1%, respectively. Patients whose tumor Met H-score values were below the median had shorter

Table 3: Association of MET IHC with other histopathological features in TMA samples (n = 196 cores)

	MET H-score med [P_{25}–P_{75}]	p-value
Histological pattern		
Acinar	35 [0–280]	0.033
Lepidic	400 [300–400]	
Solid	30 [0–400]	
Papillary	25 [0–78.5]	
Histological grade		
1	360 [97.5–400]	0.010
2	60 [0–200]	
3	30 [0–383]	
Staining pattern		
Cytoplasmic	20 [7.25–160]	0.003
Membranous	240 [40–400]	
Vimentin		
Positive	0 [0–150]	0.027
Negative	80 [50–340]	
E-cadherin		
Positive	80 [1–350]	0.003
Negative	0 [0–20]	

TMA, Tissue microarray; *IHC*, immunohistochemistry

survival times when compared with patients with above-median values, but thiswas not statistically significant (p = 0.175). Interestingly, patients with tumors showing either a predominantly cytoplasmic Met staining or expression of mesenchymal features (i.e. vimentin positivity) had shorter survival times, and these differences were statistically significant (p = 0.042 and p = 0.047, respectively). Survival curves are illustrated in Figure 6.

DISCUSSION

Lung adenocarcinoma is a morphologically heterogeneous disease. Multiple histological patterns can be identified when surgical samples are evaluated [37]. This may be due to underlying genetic heterogeneity as described for other neoplasms [38, 39] as well as for NSCLC [40, 41]. In routine clinical practice, we use biopsy or cytology samples, which contain only a small fraction of tumor, to make treatment decisions and select patients for clinical trials. In our study, patient classification by IHC could vary in up to 40% among different tumor areas.

We also corroborated that *de novo MET* amplification is a rare event, in the range of other genetic alterations such as *ROS* or *ALK* rearrangements [42]. Furthermore, although FISH positive cases showed significantly higher MET H-score values, correlation between *MET* gains and total MET protein expression was poor. We also identified cases with evident discordance between MET IHC and FISH, for which the underlying mechanism is not clear. However, it is consistent with findings from other studies [43].

The difficulty of finding the correct predictive biomarker for MET-targeted therapies may explain, at least in part, the lack of success of the two largest trials testing MET inhibitors combined with Erlotinib in NSCLC patients. The MARQUEE trial [22] selected patients with nsNSCLC histology based on data of a phase II with Tivantinib [44]. The MetLung trial [23, 33], also based on phase II data with Onartuzumab [33], performed a more restrictive selection, including only patients with +2/+3 staining in at least 50% of tumor cells. Interestingly, a molecular-based post-hoc analysis was conducted on approximately 40% of the patients (based on tissue availability) participating in the MARQUEE trial. This analysis revealed a survival benefit in those patients with high MET protein expression determined by by MetMab criteria (HR 0.7; p = 0.03) [45].

Another strategy for the development of MET inhibitors in NSCLC relies on patient selection based on *MET* gains or gene amplification. In the MARQUEE study, no statistically significant differences were observed in overall survival between *MET* amplified and non-amplified cases (HR 0.83; p = 0.34) [45]. Conversely, a subgroup analysis of the phase II study with Onartuzumab revealed a survival benefit for *EGFR* wild-type and *MET* FISH positive [15] patients receiving the combined

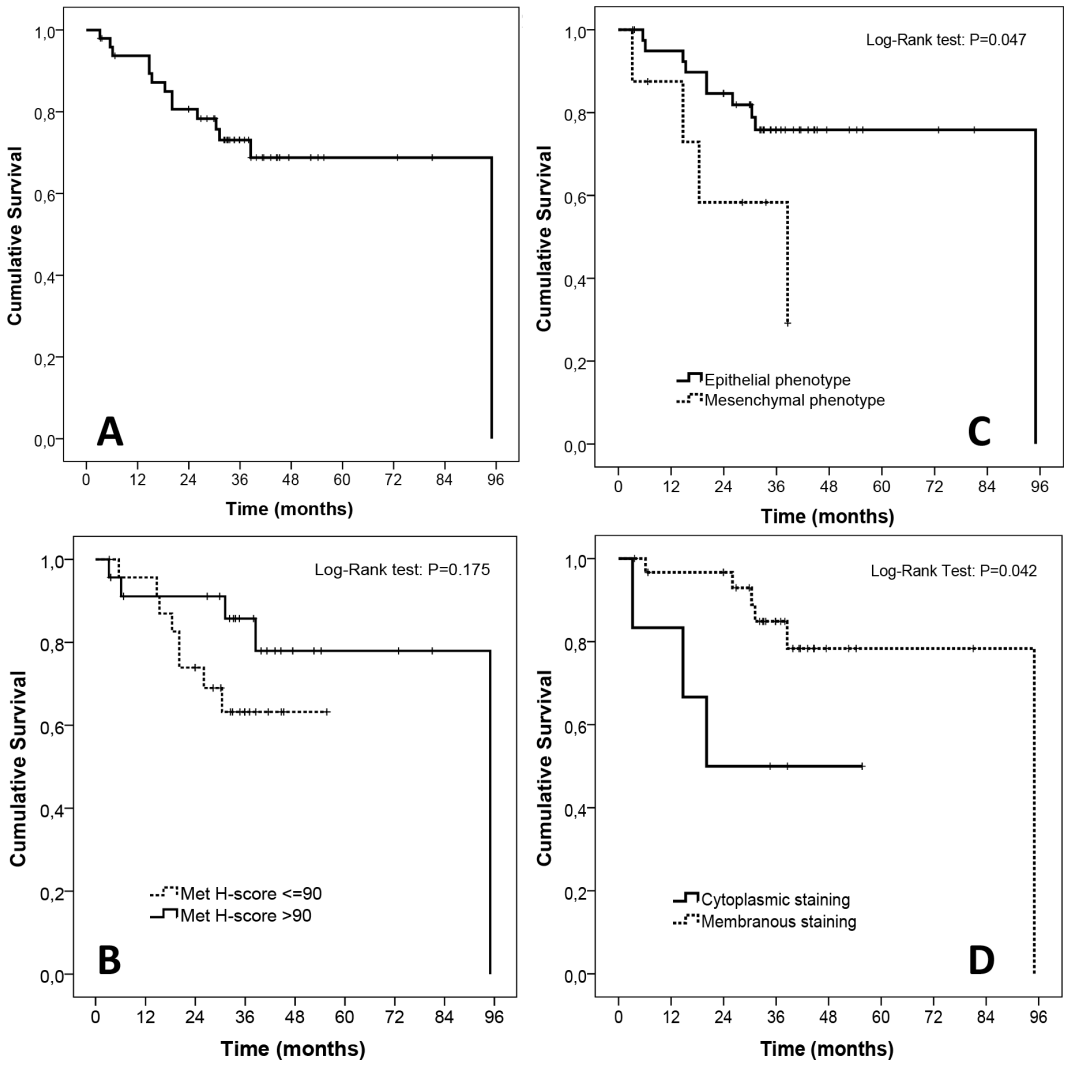


Figure 6: Exploratory survival analyses in the TMA cohort. A. Overall survival of the whole cohort (n = 47 patients, median overall survival not reached). **B.** Survival according to MetMab status. **C.** Survival according to MET staining pattern. **D.** Survival according to epithelial or mesenchymal phenotype.

treatment with Onartuzumab plus Erlotinib (HR 0.3; p = 0.06) [46]. Recently, data from a phase I/II trial with Crizotinib reported significant clinical responses in patients with *MET* amplification. Those patients with a *MET*/CEP7 ratio of ≥ 5.0 showed significantly better outcomes [36]. Although these results need to be confirmed in larger clinical trials, FISH-based criteria appear to be more adequate for patient selection. If *MET* status by FISH is less heterogeneous than MET IHC remains to be determined, as small numbers in our study (only three FISH positive cases in the TMA cohort) prevent us from drawing any robust conclusions.

Classically, it has been accepted that, after activation at the cell membrane, tyrosine-kinasereceptors (RTK) are internalized and degraded or recycled back to the membrane. However, during the last decade, preclinical evidence has emerged that highlights the role of receptor endocytosis and intracellular trafficking in RTK-mediated signaling [47–49]. In a preclinical model with immortalized bronchial cells, sustained stimulation with HGF caused a gradual displacement of c-MET receptor from the membrane to the cytoplasm [50]. Also, recent studies have associated the presence of cytoplasmic Met determined by IHC with tumor progression in patients with resected bladder cancer [51] and with poor outcome in patients with gastric adenocarcinoma [27] and mesothelioma [50]. Also, the presence of a mesenchymal phenotype, which can be an early event in NSCLC [52], has been linked to poor prognosis and metastasis development in surgically resected NSCLC [53].

Finally, our evaluation of potential surrogate markers for MET activation revealed interesting findings. Predominant cytoplasmic staining, which may translate MET pathway activation, was associated with a mesenchymal phenotype, which in turn can also be derived from MET HGF-dependent activation [34]. Although only hypothesis-generating, these results would

be concordant with these patients presenting a worse prognosis, as observed in our limited series and harbor a potential predictive value for MET inhibitor benefit.

In conclusion, our study shows that MET status is highly heterogeneous within nsNSCLC tumors. This notion challenges current techniques and criteria for selecting patients for MET-targeted therapies. Further studies are needed to accurately detect patients with MET-driven tumors.

FINANCIAL SUPPORT

This work was supported by RD12/0036/0051 /FEDER, PI13/00140/FEDER, a grant from Fundacio Marato de TV3. Ref.666/C/2013 and 2014 SGR 740. the "Xarxa de Bancs de tumors sponsored by Pla Director d'Oncologia de Catalunya (XBTC)." JA is recipient of intensification programme ISCIII.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69–90.
2. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med. 2008; 359:1367–80.
3. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, Nishiwaki Y, Ohe Y, Yang JJ, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med. 2009; 361:947–57.
4. Scagliotti G, Stahel RA, Rosell R, Thatcher N, Soria JC. ALK translocation and crizotinib in non-small cell lung cancer: an evolving paradigm in oncology drug development. Eur J Cancer. 2012; 48:961–73.
5. Camidge DR, Bang YJ, Kwak EL, Iafrate AJ, Varella-Garcia M, Fox SB, Riely GJ, Solomon B, Ou SH, Kim DW, Salgia R, Fidias P, Engelman JA, et al. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. Lancet Oncol. 2012; 13:1011–9.
6. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, Palmero R, Garcia-Gomez R, Pallares C, Sanchez JM, Porta R, Cobo M, Garrido P, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012; 13:239–46.
7. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004; 350:2129–39.
8. Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G. Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci U S A. 1987; 84:6379–83.
9. Giordano S, Ponzetto C, Di Renzo MF, Cooper CS, Comoglio PM. Tyrosine kinase receptor indistinguishable from the c-met protein. Nature. 1989; 339:155–6.
10. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009; 139:871–90.
11. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003; 4:915–25.
12. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. Nat Rev Cancer. 2012; 12:89–103.
13. Ma PC, Jagadeeswaran R, Jagadeesh S, Tretiakova MS, Nallasura V, Fox EA, Hansen M, Schaefer E, Naoki K, Lader A, Richards W, Sugarbaker D, Husain AN, et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res. 2005; 65:1479–88.
14. Takanami I, Tanana F, Hashizume T, Kikuchi K, Yamamoto Y, Yamamoto T, Kodaira S. Hepatocyte growth factor and c-Met/hepatocyte growth factor receptor in pulmonary adenocarcinomas: an evaluation of their expression as prognostic markers. Oncology. 1996; 53:392–7.
15. Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, Del Grammastro M, Sciarrotta MG, Buttitta F, Incarbone M, Toschi L, Finocchiaro G, Destro A, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol. 2009; 27:1667–74.
16. Dziadziuszko R, Wynes MW, Singh S, Asuncion BR, Ranger-Moore J, Konopa K, Rzyman W, Szostakiewicz B, Jassem J, Hirsch FR. Correlation between MET gene copy number by silver *in situ* hybridization and protein expression by immunohistochemistry in non-small cell lung cancer. J Thorac Oncol. 2012; 7:340–7.
17. Park S, Choi YL, Sung CO, An J, Seo J, Ahn MJ, Ahn JS, Park K, Shin YK, Erkin OC, Song K, Kim J, Shim YM, et al. High MET copy number and MET overexpression: poor outcome in non-small cell lung cancer patients. Histol Histopathol. 2012; 27:197–207.
18. Martens T, Schmidt NO, Eckerich C, Fillbrandt R, Merchant M, Schwall R, Westphal M, Lamszus K. A novel one-armed anti-c-Met antibody inhibits glioblastoma growth *in vivo*. Clin Cancer Res. 2006; 12:6144–52.
19. Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett. 2005; 225:1–26.

20. Catenacci DV, Henderson L, Xiao SY, Patel P, Yauch RL, Hegde P, Zha J, Pandita A, Peterson A, Salgia R. Durable complete response of metastatic gastric cancer with anti-Met therapy followed by resistance at recurrence. Cancer Discov. 2011; 1:573–9.

21. Ou SH, Bazhenova L, Camidge DR, Solomon BJ, Herman J, Kain T, Bang YJ, Kwak EL, Shaw AT, Salgia R, Maki RG, Clark JW, Wilner KD, et al. Rapid and dramatic radiographic and clinical response to an ALK inhibitor (crizotinib, PF02341066) in an ALK translocation-positive patient with non-small cell lung cancer. J Thorac Oncol. 2010; 5:2044–6.

22. Scagliotti GV, Novello S, Schiller JH, Hirsh V, Sequist LV, Soria JC, von Pawel J, Schwartz B, Von Roemeling R, Sandler AB. Rationale and design of MARQUEE: a phase III, randomized, double-blind study of tivantinib plus erlotinib versus placebo plus erlotinib in previously treated patients with locally advanced or metastatic, nonsquamous, non-small-cell lung cancer. Clin Lung Cancer. 2012; 13:391–5.

23. Spigel DR, Edelman MJ, Mok T, O'Byrne K, Paz-Ares L, Yu W, Rittweger K, Thurm H. Treatment Rationale Study Design for the MetLung Trial: A Randomized, Double-Blind Phase III Study of Onartuzumab (MetMAb) in Combination With Erlotinib Versus Erlotinib Alone in Patients Who Have Received Standard Chemotherapy for Stage IIIB or IV Met-Positive Non-Small-Cell Lung Cancer. Clin Lung Cancer. 2012; 13:500–4.

24. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008; 321:1807–12.

25. Tao Y, Ruan J, Yeh SH, Lu X, Wang Y, Zhai W, Cai J, Ling S, Gong Q, Chong Z, Qu Z, Li Q, Liu J, et al. Rapid growth of a hepatocellular carcinoma and the driving mutations revealed by cell-population genetic analysis of whole-genome data. Proc Natl Acad Sci U S A. 2011; 108:12042–7.

26. Gerlinger M, Quezada SA, Peggs KS, Furness AJ, Fisher R, Marafioti T, Shende VH, McGranahan N, Rowan AJ, Hazell S, Hamm D, Robins HS, Pickering L, et al. Ultra-deep T cell receptor sequencing reveals the complexity and intratumour heterogeneity of T cell clones in renal cell carcinomas. J Pathol. 2013; 231:424–32.

27. Ha SY, Lee J, Kang SY, Do IG, Ahn S, Park JO, Kang WK, Choi MG, Sohn TS, Bae JM, Kim S, Kim M, Kim S, et al. MET overexpression assessed by new interpretation method predicts gene amplification and poor survival in advanced gastric carcinomas. Mod Pathol. 2013; 26:1632–41.

28. Arriola E, Canadas I, Arumi-Uria M, Domine M, Lopez-Vilarino JA, Arpi O, Salido M, Menendez S, Grande E, Hirsch FR, Serrano S, Bellosillo B, Rojo F, et al. MET phosphorylation predicts poor outcome in small cell lung carcinoma and its inhibition blocks HGF-induced effects in MET mutant cell lines. Br J Cancer. 2011; 105:814–23.

29. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med. 1998; 4:844–7.

30. Salido M, Tusquets I, Corominas JM, Suarez M, Espinet B, Corzo C, Bellet M, Fabregat X, Serrano S, Sole F. Polysomy of chromosome 1 in breast cancer tumors showing an overexpression of ERBB2: a study of 15 cases using fluorescence *in situ* hybridization and immunohistochemistry. Breast Cancer Res. 2005; 7:R267–73.

31. Camidge DR, Kono SA, Flacco A, Tan AC, Doebele RC, Zhou Q, Crino L, Franklin WA, Varella-Garcia M. Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. Clin Cancer Res. 2010; 16:5581–90.

32. Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst. 2005; 97:643–55.

33. Spigel DR, Ervin TJ, Ramlau RA, Daniel DB, Goldschmidt JH Jr, Blumenschein GR Jr, Krzakowski MJ, Robinet G, Godbert B, Barlesi F, Govindan R, Patel T, Orlov SV, et al. Randomized phase II trial of Onartuzumab in combination with erlotinib in patients with advanced non-small-cell lung cancer. J Clin Oncol. 2013; 31:4105–14.

34. Canadas I, Rojo F, Taus A, Arpi O, Arumi-Uria M, Pijuan L, Menendez S, Zazo S, Domine M, Salido M, Mojal S, Garcia de Herreros A, Rovira A, et al. Targeting Epithelial-to-Mesenchymal Transition with Met Inhibitors Reverts Chemoresistance in Small Cell Lung Cancer. Clin Cancer Res. 2014; 20:938–50.

35. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Statistics Subcommittee of the NCIEWGoCD. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst. 2005; 97:1180–4.

36. Camidge DR, Ou S-HI, Shapiro G, Otterson GA, Villaruz LC, Villalona-Calero MA, Iafrate AJ, Varella-Garcia M, Dacic S, Cardarella S, Zhao W, Tye L, Stephenson P, et al. Efficacy and safety of crizotinib in patients with advanced c-MET-amplified non-small cell lung cancer (NSCLC). ASCO Meeting Abstracts. 2014; 32:8001.

37. Travis WD, Brambilla E, Riely GJ. New pathologic classification of lung cancer: relevance for clinical practice and clinical trials. J Clin Oncol. 2013; 31:992–1001.

38. Fisher R, Larkin J, Swanton C. Inter and intratumour heterogeneity: a barrier to individualized medical therapy in renal cell carcinoma? Front Oncol. 2012; 2:49.

39. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, Kamiyama M, Hruban RH, Eshleman JR, Nowak MA, Velculescu VE, Kinzler KW, Vogelstein B, et al. Distant

metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010; 467:1114–7.

40. Cancer Genome Atlas Research N: Comprehensive molecular profiling of lung adenocarcinoma. Nature. 2014; 511:543–50.

41. Chen Z, Fillmore CM, Hammerman PS, Kim CF, Wong KK. Non-small-cell lung cancers: a heterogeneous set of diseases. Nat Rev Cancer. 2014; 14:535–46.

42. Guo B, Cen H, Tan X, Liu W, Ke Q. Prognostic value of MET gene copy number and protein expression in patients with surgically resected non-small cell lung cancer: a meta-analysis of published literatures. PLoS One. 2014; 9:e99399.

43. Xiu J, Feldman R, Bender RP, Salgia R. Tumor biomarker evaluation of 6, 785 patients for combination treatment strategies in NSCLC. ASCO Meeting Abstracts. 2014; 32:e19012.

44. Sequist LV, von Pawel J, Garmey EG, Akerley WL, Brugger W, Ferrari D, Chen Y, Costa DB, Gerber DE, Orlov S, Ramlau R, Arthur S, Gorbachevsky I, et al. Randomized phase II study of erlotinib plus tivantinib versus erlotinib plus placebo in previously treated non-small-cell lung cancer. J Clin Oncol. 2011; 29:3307–15.

45. Novello S, Scagliotti GV, Ramlau R, Barlesi F, Sandler AB, Von Roemeling R. Efficacy Analysis for Molecular Subgroups in MARQUEE: a Randomized, Double-blind, Placebo-controlled, Phase 3 Trial of Tivantinib (ARQ 197) Plus Erlotinib versus Placebo plus Erlotinib in Previously Treated Patients with Locally Advanced or Metastatic, Non-squamous, Non- small Cell Lung Cancer (NSCLC). Poster presented at: World Congress on Lung Cancer; 2013 27–31; Sidney, Australia.

46. Koeppen H, Yu W, Zha J, Pandita A, Penuel E, Rangell L, Raja R, Mohan S, Patel R, Desai R, Fu L, Do A, Parab V, et al. Biomarker Analyses from a Placebo-Controlled Phase II Study Evaluating Erlotinib +/– Onartuzumab in Advanced Non-Small Cell Lung Cancer: MET Expression Levels Are Predictive of Patient Benefit. Clin Cancer Res. 2014; 20:4488–98.

47. Joffre C, Barrow R, Menard L, Calleja V, Hart IR, Kermorgant S. A direct role for Met endocytosis in tumorigenesis. Nat Cell Biol. 2011; 13:827–37.

48. Barrow R, Joffre C, Menard L, Kermorgant S. Measuring the role for Met endosomal signaling in tumorigenesis. Methods Enzymol. 2014; 535:121–40.

49. von Zastrow M, Sorkin A. Signaling on the endocytic pathway. Curr Opin Cell Biol. 2007; 19:436–45.

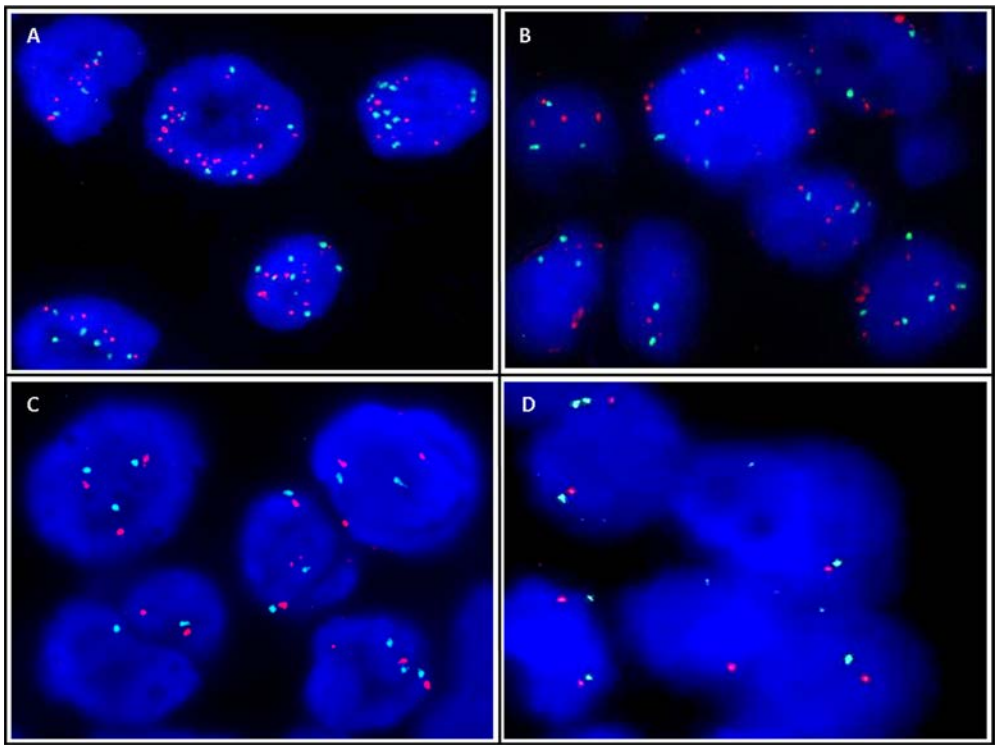
50. Levallet G, Vaisse-Lesteven M, Le Stang N, Ilg AG, Brochard P, Astoul P, Pairon JC, Bergot E, Zalcman G, Galateau-Salle F. Plasma cell membrane localization of c-MET predicts longer survival in patients with malignant mesothelioma: a series of 15 cases from the MESOPATH Group. J Thorac Oncol. 2012; 7:599–606.

51. Kluth M, Reynolds K, Rink M, Chun F, Dahlem R, Fisch M, Hoppner W, Wagner W, Doh O, Terracciano L, Simon R, Sauter G, Minner S. Reduced membranous MET expression is linked to bladder cancer progression. Cancer Genet. 2014; 207:147–52.

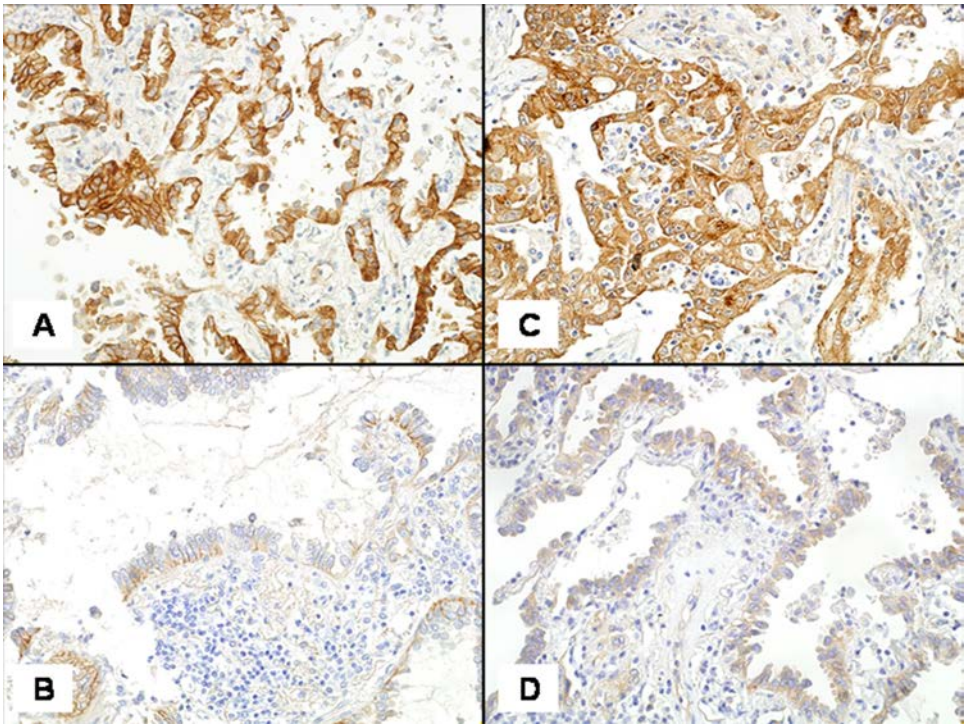
52. Prudkin L, Liu DD, Ozburn NC, Sun M, Behrens C, Tang X, Brown KC, Bekele BN, Moran C, Wistuba II. Epithelial-to-mesenchymal transition in the development and progression of adenocarcinoma and squamous cell carcinoma of the lung. Mod Pathol. 2009; 22:668–78.

53. Soltermann A, Tischler V, Arbogast S, Braun J, Probst-Hensch N, Weder W, Moch H, Kristiansen G. Prognostic significance of epithelial-mesenchymal and mesenchymal-epithelial transition protein expression in non-small cell lung cancer. Clin Cancer Res. 2008; 14:7430–7.

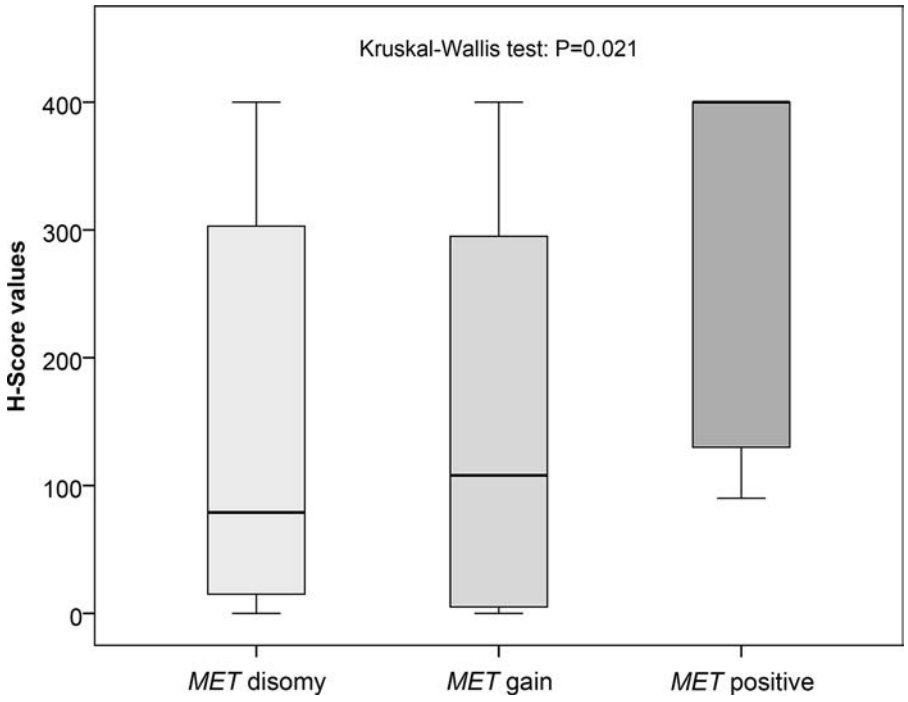
SUPPLEMENTARY FIGURES AND TABLE



Supplementary Figure 1: Different FISH signal patterns. Four tumor samples exhibiting different *MET*/CEP7 FISH signal patterns: **A.** *MET* positive nuclei showing a *MET*/CEP7 ratio ≥ 2 (truly amplified). **B.** *MET* positive nuclei showing a *MET* ≥ 5 (high polysomy). **C.** *MET* negative nuclei showing ≥ 2.5 *MET* copies (classified as *MET* gain). **D.** *MET* negative disomic pattern.



Supplementary Figure 2: Different MET staining patterns at 40x. A. strong (+4) predominantly membranous pattern (H-score: 400). **B.** weak (+1) predominantly membranous pattern (H-score: 15). **C.** strong (+4) predominantly cytoplasmic pattern (H-score: 400). **D.** weak (+1) predominantly cytoplasmic pattern (H-score: 60).



Supplementary Figure 3: MET IHC H-score differences among FISH categories. *MET* FISH positive cases (high polysomy and truly amplified) have higher MET H-scores.

		CORE B		CORE C		CORE D	
		Memb.	Cytopl.	Memb.	Cytopl.	High	Cytopl.
CORE A	Memb.	24	3	26	2	23	1
	Cytopl.	3	0	5	0	1	2
Percent change		20% (6/30)		21.2% (7/33)		0.08% (2/25)	

Supplementary Figure 4: Heterogeneity in MET IHC staining pattern among different tumor cores. Staining pattern was heterogeneous among tumor cores. No case showed cytoplasmic staining in all four cores (data not shown). Abbreviations: *Cytopl*., Cytoplasmic; *Memb*., Membranous.

Supplementary Table 1: Association between MET IHC and *MET* FISH

	MET H-score med [P_{25}–P_{75}]	*p*-value	*MET* gene copies med [P_{25}–P_{75}]	*p*-value
MET IHC				
MET high (n = 55)	350 [253–400]	<0.001	3 [2–4]	0.682
MET low (n = 60)	23 [1.38–79.75]		3 [2–3.02]	

IHC, immunohistochemistry

Article 2. Heterogeneity of tumor and immune cell PD-L1 expression and lymphocyte counts in surgical NSCLC samples

Authors: David Casadevall, Sergi Clavé, Álvaro Taus, Max Hardy-Werbin, Pedro Rocha, Marta Lorenzo, Silvia Menéndez, Marta Salido, Joan Albanell, Lara Pijuan, Edurne Arriola

BIBLIOGRAPHIC REFERENCE:

PMID: 28549836

DOI: 10.1016/j.cllc.2017.04.014

5. DISCUSSION

5.1. Heterogeneity of biomarker expression

In the two studies presented here, we have demonstrated a heterogeneous distribution of molecular and histologic biomarkers in NSCLC samples. This could represent a challenge for biomarker-based patient classification, which is a crucial step in therapeutic decision-making in this disease. The biomarkers included in our studies and the criteria to evaluate them were chosen based on their use in contemporary clinical trials of targeted therapies. In the first study, in which we looked at the status of MET, we found that 20-40% of patients could be misclassified using MET IHC. Regarding MET FISH status, although the number of positive cases was low (6.8%), 33% (4 of 12) cores analyzed in positive cases presented a negative FISH result. In the second study, in which we looked at PD-L1 expression in tumor cells and in tumor-infiltrating lymphocytes, we also found biomarker heterogeneity. 10% of ADCs and 19% of SCCs could be misclassified based on tumor PD-L1 expression, while the rates of misclassification considering immune cell PD-L1 positivity were 26% and 17% in ADC and SCC, respectively. Similar to what we observed with *MET* amplification, *PDL1* amplification was rare, but 36.4% (4 of 11) of the *PDL1* amplified cases harbored FISH negative areas. Aside from other biological and technical aspects, such heterogeneity in biomarker expression could partly explain their shortcomings as predictive markers of targeted therapy efficacy. Here, I will first discuss our results in the context of the current evidence regarding MET and PD-L1 heterogeneity, followed by examine the current evidence regarding IHC and FISH methods for MET and PD-L1 assessment and their correlation with other clinical, histopathological and molecular variables. Third, I will discuss the strengths and limitations of our studies. Finally, I will make a brief overview of what is currently known regarding NSCLC ITH and the possible approaches to address it in clinical practice.

There are not many reports that have directly addressed MET intratumor heterogeneity in NSCLC. Regarding protein expression, the first proof of the existence of spatially heterogeneous MET expression in NSCLC samples comes from the design of the Metmab IHC positivity criteria. From the beginning, the designated cut-off for MET positivity (≥50% of tumor cells with moderate or strong staining) was intended to account for the observed heterogeneity of MET expression (see Image 1 on the following page)[125,161]. This already suggests that the study of small tumor areas could fail to assess overall MET protein status, as we found in our study. A recently published study by Lapère et al further supports our findings of intratumor heterogeneity regarding MET protein expression. Employing the Metmab criteria on whole-tissue slides of 62 surgically treated NSCLC patients, they independently scored different tumor areas. They identified a high level of intratumor heterogeneity (defined as

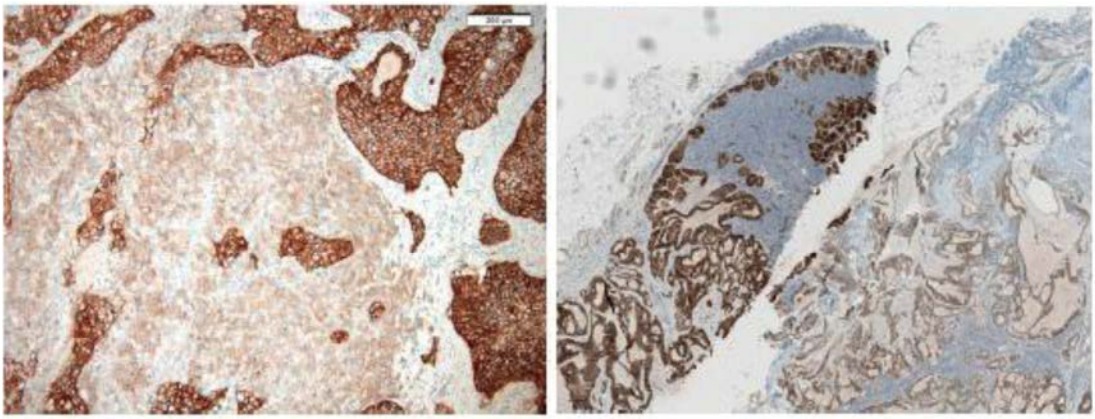


Image 1: samples from a post-hoc biomarker design study[125] of the phase II trial with onartuzumab showing heterogeneous MET expression within tumor samples. Reproduced with permission from the author.

≥30% of tumor cells displaying +2/+3 staining in an overall Metmab negative sample or the same proportion of 0/+1 cells in a sample scored as Metmab positive) in 30-40% of tumor specimens. Furthermore, they examined more than one tissue sample per patient and discordant scoring between samples was observed in 10-12% of cases[162].

Regarding the assessment of *MET* gene status by in situ hybridization techniques, there has been a lack of consensus about which is the optimal cut-off to define MET positivity (Table 1). In this context, however, some criteria such as the UCCC (University of Colorado Cancer Center) take into account the existence of small cell clusters with high concentration of *MET* signals, counting a tumor sample as positive if such cells represent ≥ 10% of the sample and further suggesting the existence of intratumor heterogeneity of MET status. An elegant study by Schildhaus et al looked at MET FISH status in a large cohort (n=693) of NSCLC samples and was able to identify several patterns of *MET* signal distributions, including cases with clearly defined signal heterogeneity[163]. Notably, the most promising FISH criterion for defining MET-driven tumors comes from clinical trials with crizotinib, in which patient selection was based on mean *MET*/CEP7 ratio[112,116,164]. Some patients with clearly amplified tumor areas (see Image 2 on the following page) may be missed by this criterion if only small tumor areas are evaluated. Furthermore, if the aforementioned patterns of heterogeneous MET signal distribution[163] also predict benefit from MET-targeting drugs is still unknown.

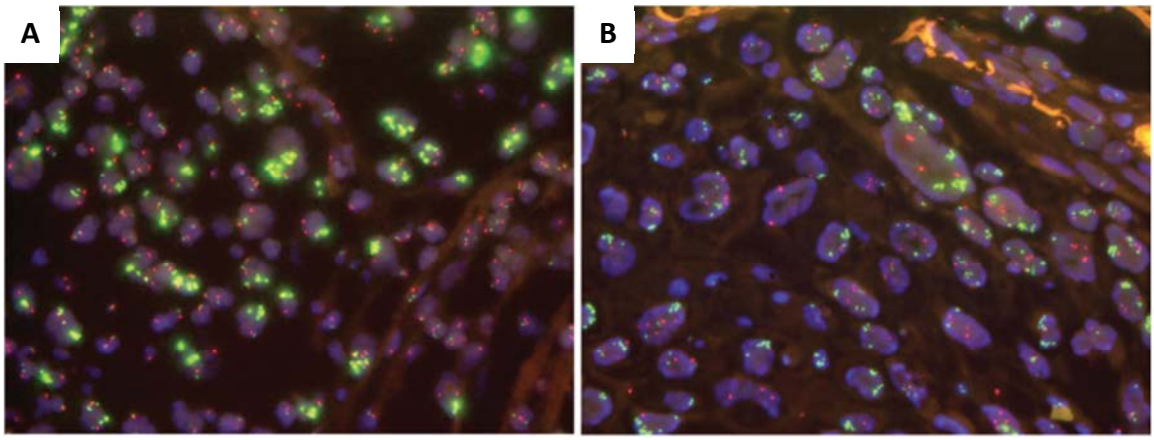


Image 2: samples from the study by Schildhaus et al[163] showing heterogeneous distribution of *MET* amplified cells. **A:** broadly heterogeneous MET signal (green) distribution. **B:** clusters or "hot-spot" areas *MET*-amplified cells. Reproduced with permission from the author.

Contrary to what happens with MET, intratumor heterogeneity of PD-L1 expression has been the subject of extensive research. Of note, this research has been challenged by the multiple antibody clones available for PDL1 assessment (discussed further below). Four studies have addressed the intratumor heterogeneity of PD-L1 expression in NSCLC using the same antibody clone we used in our study (SP142 Rabbit clone, Ventana)[165–168]. Two studies used quantitative immunofluorescence to assess PDL1 positivity in tumor and stromal compartments in whole tissue sections of NSCLCs, reporting a significant degree of variability of PD-L1 expression in both compartments between different tumor areas (Image 3)[165,166].

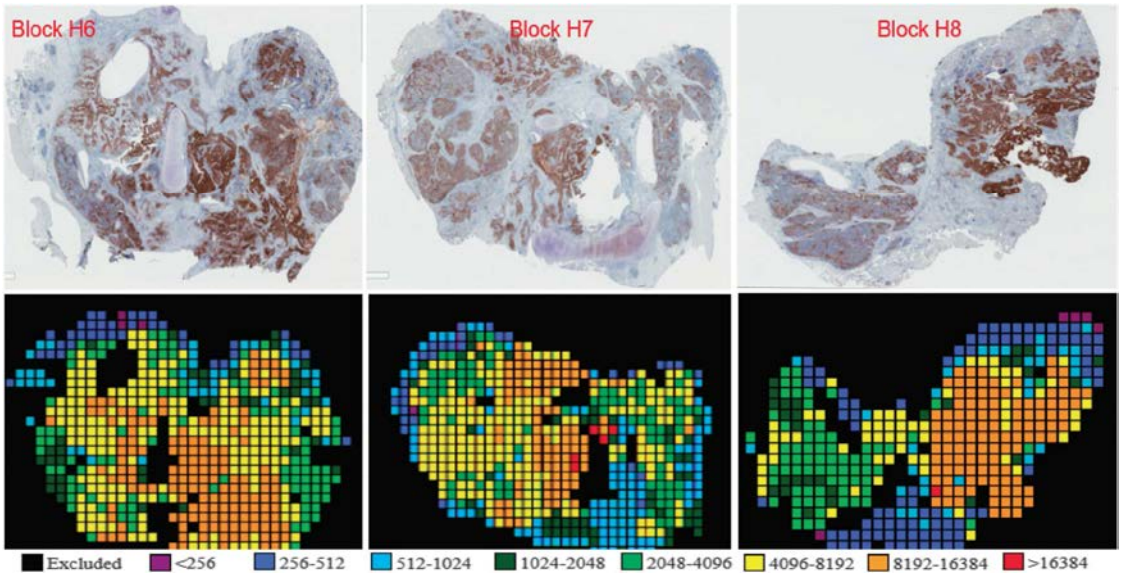


Image 3: Immunohistochemistry and quantitative immunofluorescence of three tissue blocks shows variability of PDL1 intensity among different field of views[166]. Reproduced with permission from the author.

Another study, led by Ilie et al, assessed tumor and immune cell scoring using currently accepted cut-offs[169] in matched biopsies from resected NSCLC samples. They found that biopsy samples underestimated the PD-L1 positivity rates of surgical samples in all cases, mainly due to a lack of immune cell representation in biopsy samples (75% of total discordant cases). Looking only at tumor cell positivity, the remaining 25% were negative in the biopsy sample but showed positive tumor cell staining in the surgical specimen[168]. Finally, a study led by Gniadek et al analyzed the prevalence of PD-L1 tumor cell staining in TMA cores (3-4 cores per patient), evaluating each core individually and employing different cut-offs for positivity. They found significant discordances of PD-L1 scoring between cores, which they attributed to spatially heterogeneous PD-L1 expression after examining the corresponding whole tissue slides. According to their study, the diagnostic sensitivity based on a single tumor sample and using the 50% tumor cell PD-L1 positivity cut-off would be 85% in adenocarcinomas and 95% in squamous cell carcinomas, with near perfect (≈ 99%) specificity[167].

A further study conducted by Li et al using the 22C3 antibody (Dako) elegantly showed that TMA samples could underestimate or overestimate PD-L1 status in approximately 50% of cases, when compared with whole tissue sections from surgically resected NSCLC specimens (Image 4). Interestingly, the rate of discordance varied with the different cut-offs used, being lowest for PD-L1 negative cases (18.4%)[170].

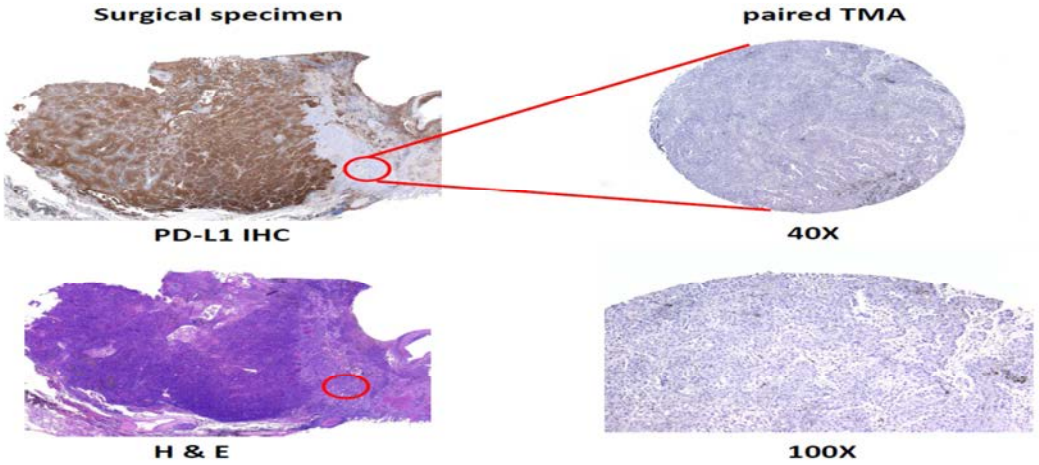


Image 4: example of PD-L1 status underestimation in a non-representative TMA core taken from a surgical specimen with clear PD-L1 positive areas[170]. Reproduced with permission from the author.

In contrast, an additional study conducted by Kitazono et al reported a high concordance regarding PD-L1 status between biopsy and surgical samples. Of note, however, he used an antibody that is not validated in clinical trials and only studied one threshold (any PD-L1

staining considered positive). Interestingly, the studies conducted by Gniadek[167] and Li[170] used TMAs to estimate the extent of PD-L1 heterogeneity across tumor samples, thus employing a similar strategy to that of our studies. One of the caveats of this strategy may the size of the TMA cores, since sometimes the tumor area within diagnostic tissue samples can be presumably be larger compared with that of the core. In our study, we sampled 1mm-diameter cores, while Gniadek and Li analyzed cores that measured 0.6 and 2mm, respectively. Furthermore, Li et al employed a different PD-L1 antibody clone compared with that used by us and Gniadek. Despite these methodological differences, both authors reached the same conclusion as us, namely that the expression of PD-L1 is heterogeneous and that the analysis of small tumor samples may lead to patient misclassification.

Another class of heterogeneity that can potentially affect patient classification is the discordant expression of biomarkers between primary tumors and their metastases. Heterogeneous expression of PD-L1 between different tumor sites has been reported for patients with multifocal lung cancer[171] as well as between primary tumors and corresponding nodal metastases[172]. Also, Discordance of tumor PD-L1 expression between primary lesions and distant metastases has been reported, ranging from 12 to 14% and being primarily due to a lower PD-L1 expression in metastases compared to their matched primary tumors[173,174].

Taken together, our results and those of the above-mentioned studies suggest that MET and PD-L1 be expressed heterogeneously within tumors and supports the hypothesis that this heterogeneity can impact biomarker assessment. Patient misclassification due to biomarker heterogeneity might potentially explain the failure of the biomarker/s to predict benefit from targeted therapies.

5.2. Discordance between MET IHC and FISH and definition of MET-driven NSCLC

With respect to MET evaluation, one of the main issues that have arisen is the role of immunohistochemistry in identifying patients likely to benefit from MET-targeted treatment. The studies that have performed IHC-based patient selection have failed to prove a therapeutic benefit in their experimental arms. In the onartuzumab phase III trial MetLung, patients were selected by MET IHC positivity (Metmab criteria). Strikingly, patients included in the onartuzumab+erlotinib arm experienced a higher incidence of deaths and a worse overall survival compared with patients in the placebo+erlotinib arm (52% of deaths vs. 46%; 6.8 months of median overall survival vs. 9.1)[124]. In a recently published phase I/II trial that evaluated the addition of MET inhibitor emibetuzumab to erlotinib in *EGFR*-mutant patients

with acquired resistance to EGFR TKI, patients were selected by positive IHC expression of MET (moderate intensity staining in ≥10% of cells). However, no benefit was observed in the emibetuzumab arm, even when the threshold of MET positive cells was risen to ≥60% according to a preplanned analysis[175]. In early-phase clinical trials of Met tyroskine kinase inhibitor capmatinib (INC280), patients receiving capmatinib+erlotinib that presented high MET protein expression by IHC (defined as H-score ≥150 or IHC 2+ or 3+) have shown response rates of 19-29%, compared with 18-19% in the overall study population[176,177]. Although future trial results are awaited, these preliminary findings are not encouraging with regard to the predictive value of MET IHC positivity.

In contrast with MET protein expression, *MET* gene alterations appear to be a more solid biomarker of MET-targeted therapy benefit. Interestingly, *MET* amplification was not prognostic in a post-hoc biomarker study of the onartuzumab phase II trial, suggesting that this effect might be confined to treatment with MET tyrosine kinase inhibitors[125]. Moreover, the proper cut-off of *MET* gene gain that defines *MET* amplification (at least to a degree that predicts significant benefit from MET TKIs) is still to be defined. Currently, the most promising cut-off is a *MET*/CEP7 ratio of ≥5, as can be interpreted from recent experience with crizotinib[112,115,116]. However, the prevalence of high-level amplification of *MET* has been reported to be 0.3-1.2%[164,178,179], which may hamper trial design and subsequent drug approva, as well as clinical impact. In this context, the role of absolute *MET* copy number gain has yet to be unveiled. In one of the aforementioned capmatinib trials in which patients were selected based on MET protein expression or *MET* gene gain, five out of eight patients with ≥5 mean MET copy number presented significant responses (63% overall response rates)[177]. In a second capmatinib trial, in which only MET positive *EGFR*-mutant patients were included, a mean *MET* copy number of ≥6 was associated with a 30% overall response rate[176]. The role of the *MET*/CEP7 ratio in these two trials was not reported.

Another finding that suggests a predictive role of *MET* gene alterations is the recent discovery of *MET* exon 14 mutations that affect the juxtramembrane domain of the receptor, impairing its ubiquitin-mediated degradation and ultimately leading to sustained MET signaling. These mutations occur in around 3% of NSCLC and have been shown to partially overlap with *MET* amplification. In this setting, crizotinib has also shown promising activity[110,113–116]. Furthermore, two patients with *MET* exon14 mutations included in one of the capmatinib trials presented a partial response[177]. As for high-level *MET* amplifications, these findings need to be confirmed in larger clinical trials.

In our first study, four patients (3.4%) showed high *MET* copy numbers (mean ≥5) and four additional patients (3.4%) showed true *MET* amplification (*MET*/CEP7 ratio ≥2), of which one had high-level amplification (*MET*/CEP7 ratio ≥2). These numbers are in accordance with recent genomic analyses of lung ADC[61,164,178,179]. Of note, we did not assess the prevalence of *MET* mutations, since it did not bear a clear clinical significance at the time. Furthermore, although MET FISH positive cases showed significantly higher MET protein expression in our study, we failed to find a statistical correlation between *MET* gene copy number and MET protein expression when both were considered as continuous variables. This is also similar to what other studies have reported, as depicted in Table 2. Therefore, among the possible causes leading to initial clinical trial failure in trials with MET-directed therapies, evidence suggests that MET IHC is an inappropriate parameter for identifying MET-dependent tumors. Indeed, when MET tyrosine residue phosphorylation has been studied as a surrogate for MET pathway activation, it does not always correlate with either MET protein overexpression or to *MET* amplification[180,181]. Also, as MET pathway activation can occur in a variety of cellular contexts and in response to several different stimuli, MET IHC might represent an adaptative cellular state, whereas MET amplification may more rightly represent MET-driven tumors[72,90]. Interestingly, however, current molecular evidence suggests that not every degree of *MET* gain represents a driver alteration. Thus, high-level amplifications might identify truly MET-driven tumors, while lower level *MET* gains could represent passenger alterations, thus explaining treatment failure in populations selected by less stringent genomic criteria. Indirect evidence of this is found in studies of parallel analysis of known drivers in large cohorts of NSCLC[164,182]. Such studies show that low level *MET* gains overlap with other tumor drivers, while the rate of overlap decreases as the cut-off for MET amplification increases. Thus, it is tempting to hypothesize that tumors low level MET gains likely have other "dominant" driver alterations and therefore do not respond to MET-directed therapies.

5.3. *PDL1* amplification and PD-L1 IHC assay harmonization

Contrary to *MET* gene alterations, amplifications of the *PDL1* gene have not been routinely examined in trials with PD-1/PD-L1 inhibitors in NSCLC. In our study, we detected *PDL1* amplifications in 11 patients (7.8%), using a *PDL1*/CEP9 ratio of ≥2. This rate is slightly higher than the 3-5% rate reported in other series using similar criteria[183–186]. Furthermore, *PDL1* amplification has been generally associated with increased PD-L1 protein expression by IHC in NSCLC tissue cell lines[183,185,186]. In our study, however, one third of the *PDL1* amplified cases lacked tumor PD-L1 expression. Moreover, in four out of eight of amplified ADC, amplification was observed only in one of the cores (i.e. *PDL1* amplification was heterogeneous in 50% of

the cases). In contrast, no discordance in *PDL1* status was observed among the three amplified SCC cases. To our knowledge, our study is the first to specifically address the heterogeneity of *PDL1* amplification in NSCLC.

PD-L1 expression cannot be considered a reliable predictive marker of response to anti-PD-1/PD-L1 therapies, since patients with high PD-L1 expression can be treatment-refractory and good responses may be observed in PD-L1 negative populations. However, it may serve as a good enrichment marker to select a population that will have high chances of benefiting from these agents. Nevertheless, population enrichment may vary according to which anti-PD-1/PD-L1 drug needs to be administered, as each one needs a specific diagnostic assay and uses a particular definition for PD-L1 positivity (Table 3A-D). Currently, no "gold-standard" assay exists that suffices for selecting patients for more than one anti-PD-1/PD-L1 therapy, which forces pathologists to be familiar with multiple antibodies and cut-offs that often require specific training, ultimately raising clear feasibility and additional cost issues for cancer immunotherapy application.

Several efforts have been undertaken to estimate inter-assay variability and harmonize results of the available PD-L1 antibody clones. The first effort to help pathologists and clinicians has been carried out by the International Association for the study of Lung Cancer (IASCL). They have published a comprehensive atlas for PD-L1 immunohistochemical evaluation that is readily accessible online and free to download (https://www.iaslc.org/publications/iaslc-atlas-pd-l1-testing-lung-cancer). Also, several studies have been conducted, aimed at comparing the different PD-L1 antibody clones used in the clinic and in clinical trials of emergent anti-PD-1/PD-L1 drugs[187–196]. In general, these studies have reported a good inter-assay correlation regarding the evaluation of positive tumor cells. The greatest concordance rates have been observed between the 22C3 and the SP263 clones. However, inter-assay variability regarding PD-L1 immune cell positivity among tested antibody clones is remarkably higher than that found for PD-L1 tumor cell expression. Furthermore, consistently among studies, the SP142 clone (Ventana) stains fewer tumor cells compared with the remaining antibodies. This might be relevant when interpreting the results of our study, because we observed a relatively low ratio of tumor cell PD-L1 positivity. Specifically, this could partially explain why one third of *PDL1* amplified cores showed no tumor PD-L1 expression. Furthermore, we cannot rule out the possibility that heterogeneity rates could differ significantly (for both tumor and immune cell staining) from those observed with the SP142 clone if we had used an alternative antibody clone.

Finally, there has been some concern regarding the adequacy of cytology samples for evaluating PD-L1 status. Recently, Lerner et al reported a trend towards higher PD-L1 positivity rates when analyzing PD-L1 in fine-needle aspiration tissue blocks compared with tissue biopsies[197]. In contrast, Skov et al reported high concordance between cytology and matched histology specimens employing two different PD-L1 antibody clones. Thus, the suitability of cytology samples for assessing PD-L1 positivity will hopefully be established in future studies.

Despite all these efforts, a specific antibody clone with its corresponding diagnostic platform and positivity cut-off must be used depending on which drug needs to be administered to the patient. Future results of the phase II stage of the blueprint initiative, a collaborative, prospective, large-scale comparison of currently available PD-L1 clones, are awaited. Perhaps, another approach could be to perform a retrospective validation of multiple PD-L1 clones in available clinical trial samples.

5.4. Immune cell PD-L1 positivity and T-cell tumor infiltration

Several investigations strongly suggest that the tumor immune microenvironment shapes tumor biology and clinical behavior[198–200]. In our study, we performed digital quantitation of CD3+ and CD8+ lymphocytes within each TMA core. Interestingly, we found an association between lymphocyte density and PD-L1 positivity both in immune cells and tumor cells. Colocalization of PD-L1 expression with lymphocytic infiltration was reported in initial studies of predictive biomarkers with checkpoint inhibitors, lymphocytic infiltration may be itself a predictive marker of treatment efficacy[138,139]. This is in concordance with an adaptive immune response and therefore intuitively represents a favorable setting for immune-checkpoint blockade. However, since our study was not aimed to assessing lymphocytic density and therefore this aspect was not taken into account for TMA core selection, this association must be evaluated with caution. Furthermore, ADC specimens presented significantly higher CD3+ cells compared to SCC, while CD8+ were similar between the two histologic subtypes. We are not aware that this has been previously reported. This suggests the existence of different immune microenvironments in ADC compared to SCC and opens a possible future research avenue.

Finally, we did not find a prognostic role neither for CD3+ nor CD8+ counts. The latter stands in contrast with several publications that report a positive prognostic effect of high density of tumor-infiltrating lymphocytes in NSCLC[201–204]. However, it has been also reported that this

association might vary according to sampling strategy[205]. Therefore, the role of the multiple characteristics of the tumor microenvironment in patient prognostic and response to targeted therapy warrants further study.

5.5. Strengths and limitations of the studies

The main strength of our study lays on the specific design of the TMA for the study of heterogeneity within tumor areas. Patient inclusion was performed prospectively, based only on tissue availability. The inclusion is ongoing, allowing for the expansion of the current cohort and the study of additional biomarkers in the future. Furthermore, the core-by-core analyses performed in both studies, in which each tumor core was considered as an individual sample upon which several biomarker studies were performed, allowed for a detailed study of potential associations between histopathological and molecular variables. Moreover, the criteria and methods used to assess biomarker status were chosen according to contemporary relevant research, which facilitates the external comparison of our results.

One of the purposes of the studies was to estimate the potential impact of the discordances in biomarker expression between different tumor areas on patient classification. Thus, by analyzing TMA cores, we aimed at mimicking the situation of our daily clinical practice, in which the area of tumor tissue upon which biomarker studies are performed is usually small. Our TMA cores had a diameter of 1mm. Although this size is larger than the usual 0.6mm employed in most TMA studies, it may overestimate the impact of intratumor heterogeneity in biomarker-based tumor classification. Thus, we cannot exclude the possibility that sampling larger tumor areas would somewhat alter our results.

Other general limitations of our studies were the relatively small size of the analyzed cohorts. In the second study, analyzing more than two tumor areas per sample would have increased study robustness regarding the estimation of true intratumor PD-L1 heterogeneity. Moreover, biomarker evaluation was performed on surgical samples, whereas the evaluation of histologic and molecular biomarkers for targeted therapy in NSCLC is only relevant in the advanced disease setting. In this context, none of the patients included in the studies was treated with specific therapies directed against the biomarker of interest, thus precluding us from extracting further conclusions regarding the clinical impact of ITH on targeted therapy response.

In the first study, we did not perform *MET* mutation analysis, since it was not considered a relevant biomarker of therapeutic response at the time of study design. Therefore, we were not able to assess the intratumor heterogeneity of *MET* mutations.

In the second study, one potential limitation was the lack of another antibody clone to assess PD-L1 heterogeneity. Of note, the clone that we employed is known to stain fewer cells in the tumor compartment compared to other antibodies. Furthermore, while tumor cores were selected based on spatial distance and morphological aspects (the latter criterion was only applied in adenocarcinomas), the distribution of tumor stroma and of lymphtocytic infiltration was not taken into account. Therefore, the rates of PD-L1 heterogeneity in lymphocytes have to be interpreted with caution.

5.6. Current challenges for addressing ITH in biomarker studies

The evidence of ITH challenges the clinical significance of the molecular classification of tumors put forward by most of the latest NGS studies. It questions the concept of "one tumor equals one driver" and therefore represents a challenge for personalized medicine. Most importantly, ITH is being postulated as a major cause of therapeutic failure, as it increases tumor adaptability to internal and external pressures and probably provides the tumor with the necessary means for metastatic spread. Thus, it needs to be addressed and requires the development of reliable experimental models to study its mechanisms.

One of the underlying causes of ITH is genomic instability, which creates the optimal setting in which clonal selection can occur. Additionally, the microenvironment likely plays an important role in shaping and promoting ITH and mediating therapeutic resistance. Moreover, non-cell-autonomous interactions between different subclones promoted by ITH may increase tumor fitness. Conceivably, most of these elements of ITH may offer opportunities for targeted therapy. First, the drivers of genomic instability may be exploited, either by inhibiting it and thus limiting the development of ITH or by stimulating it and generating situations of synthetic lethality. Second, targeted therapies against pro-tumorigenic elements of the tumor microenvironment are being actively researched, the promising efficacy of which has been already demonstrated in experimental models. Third, clonal interactions that are relevant for tumor fitness or therapeutic resistance also could be hypothetically targeted. Finally, this approach may be useful as more biological traits and drivers of ITH are uncovered, providing hope for the future of cancer patients.

An important aspect of ITH is that the underlying clonal dynamics vary during tumor evolution. Therefore, strategies to monitor ITH and thus perform optimal biomarker analyses have to be implemented. Of these, the most promising strategy is the serial tissue sampling using liquid biopsies. Analysis of cell-free DNA and circulationg tumor cells may provide with important information about clonal architecture in a minimally invasive manner. Another plausible approach is the development of molecularly targeted imaging techniques.

The strategies necessary to overcome ITH must take into account that it's a dynamic process. This is of especial relevance, since the only way we had to evaluate tumor dynamics was, until recently, the performance of multiple tissue biopsies, to which NSCLC patients are rarely amenable. Ultimately, however, experimental models have to be perfected and multi-region sequencing experiments must be conducted to discover the evolutionary patterns of ITH within and across cancer types. Currently available data already provides evidence for the existence of parallel evolution affecting spatially and molecularly distinct tumor subclones, suggesting the existence a relatively limited number of drivers and constraints for tumor evolution. Hopefully, the characterization of these phenomena through iterative experiments will allow us to decipher common pathways of tumor evolution and design therapeutic strategies based on evolutionary predictions.

6. CONCLUSIONS

1. Intratumor heterogeneity of MET and PD-L1 was observed both at the protein and at the gene level in surgical NSCLC samples.

2. According to MET IHC, 20-40% of patients could be misclassified depending on the sampled tumor TMA area. 33.3% of the cores obtained from FISH positive patients presented a FISH negative result.

3. In adenocarcinomas, 10.1% or 29.6% of tumors could be misclassified according to PD-L1 tumor cell and lymphocyte positivity, respectively. For squamous cell carcinoma samples, these rates were 19.1% and 17.4%, respectively. *PDL1* amplification was homogeneous in squamous cell carcinoma specimens was homogeneous. In contrast, 50% of *PDL1*-amplified adenocarcinoma patients showed gene amplification in only one of the two analyzed tumor areas.

4. We found that 47.8% of patients presented MET IHC positivity, while 6.8% had a MET FISH positive result, of which 3.4% had true *MET* amplification and one patient (0.8%) had high-level MET amplification (*MET*/CEP7 ratio ≥ 5).

5. FISH positive samples presented significantly higher MET IHC H-scores. However, 50% (2/4) of the cores with true *MET* amplification had a low MET expression. Furthermore, we found no association between *MET* gene dosage and MET H-score.

6. PD-L1 positivity in ≥1% of tumor cells or ≥1% immune cells was observed in 16.8% and 27.8% of the cases, respectively, while *PDL1* amplification was present in 7.6% of the study population.

7. With a ≥5% cut-off for defining PD-L1 positivity, we found a significant association between *PDL1* amplification and tumor cell (but not immune cell) positivity. However, 33.3% of cores with *PDL1* amplification lacked tumor PD-L1 staining.

8. Neither MET nor PD-L1 positivity were associated with any clinical variables such as age, sex, smoking history or stage in any of the studies. Moreover, *PDL1* amplification and PD-L1 positivity in tumor cells or immune cells were not associated with histological subtype.

9. MET IHC expression was associated with a lepidic predominant growth pattern and a low tumor grade, and PD-L1 tumor cell positivity was associated with *KRAS* mutations.

10. PD-L1 tumor and immune cell positivity were associated with CD3+ and CD8+ lymphocytic infiltration. In contrast, *PDL1* amplification was not associated with differences neither in CD3+ nor CD8+ counts.

11. Our findings suggest the existence of intratumor heterogeneity of MET and PD-L1 expression in NSCLC, which affects biomarker-based patient classification.

7. REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012: Global Cancer Statistics, 2012. CA Cancer J Clin 2015;65(2):87–108.

2. Cheng TYD, Cramb SM, Baade PD, Youlden DR, Nwogu C, Reid ME. The international epidemiology of lung cancer: Latest trends, disparities, and tumor characteristics. J Thorac Oncol 2016;11(10):1653–71.

3. Galceran J, Ameijide A, Carulla M, et al. Cancer incidence in Spain, 2015. Clin Transl Oncol 2017;1–27.

4. Spiro SG, Gould MK, Colice GL, American College of Chest Physicians. Initial Evaluation of the Patient With Lung Cancer: Symptoms, Signs, Laboratory Tests, and Paraneoplastic Syndromes. Chest 2007;132(3):149S–160S.

5. Vansteenkiste J, Crin? L, Dooms C, et al. 2nd ESMO consensus conference on lung cancer: Early-stage non-small-cell lung cancer consensus on diagnosis, treatment and follow-up. Ann Oncol 2014;25(8):1462–74.

6. Sobin LH, Gospodarowicz MH, Wittekind C, editors. TNM Classification of Malignant Tumours, 7th Edition. Wiley-Blackwell; 2011.

7. Goldstraw P, Chansky K, Crowley J, et al. The IASLC lung cancer staging project: Proposals for revision of the TNM stage groupings in the forthcoming (eighth) edition of the TNM Classification for lung cancer. J Thorac Oncol 2016;11(1):39–51.

8. Gould MK, Kuschner WG, Rydzak CE, et al. Test performance of positron emission tomography and computed tomography for mediastinal staging in patients with non-small-cell lung cancer: a meta-analysis. Ann Intern Med 2003;139(11):879–92.

9. Herder GJM, Kramer H, Hoekstra OS, et al. Traditional Versus Up-Front [^{18} F] Fluorodeoxyglucose–Positron Emission Tomography Staging of Non–Small-Cell Lung Cancer: A Dutch Cooperative Randomized Study. J Clin Oncol 2006;24(12):1800–6.

10. Eberhardt WEE, De Ruysscher D, Weder W, et al. 2nd ESMO Consensus Conference in Lung Cancer: locally advanced stage III non-small-cell lung cancer. Ann Oncol 2015;26(8):1573–88.

11. Sehgal IS, Dhooria S, Aggarwal AN, Behera D, Agarwal R. Endosonography Versus Mediastinoscopy in Mediastinal Staging of Lung Cancer: Systematic Review and Meta-Analysis. Ann Thorac Surg 2016;102(5):1747–55.

12. De leyn P, Dooms C, Kuzdzal J, et al. Revised ests guidelines for preoperative mediastinal lymph node staging for non-small-cell lung cancer. Eur J Cardio-thoracic Surg 2014;45(5):787–98.

13. Novello S, Barlesi F, Califano R, et al. Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016;27(Supplement 5):V1–27.

14. Ettinger DS, Wood DE, Aisner DL, Akerley W, Bauman J. Non-small cell lung cancer. Natl Compr Cancer Netw 2017;

15. Fan J, Wang L, Jiang G-N, Gao W. Sublobectomy Versus Lobectomy for Stage I Non-Small-Cell Lung Cancer, A Meta-Analysis of Published Studies. Ann Surg Oncol 2012;19(2):661–8.

16. Yacoub WN, Meyers BF. Surgical resection in combination with lung volume reduction surgery for stage I non-small cell lung cancer. Semin Thorac Cardiovasc Surg 2010;22(1):38–43.

17. Evison M, Clive A, Castle L, et al. Resectable clinical N2 non-small cell lung cancer; what is the optimal treatment strategy? An update by the British Thoracic Society Lung Cancer Specialist Advisory Group. J Thorac Oncol 2017;

18. Curran WJ, Paulus R, Langer CJ, et al. Sequential vs Concurrent Chemoradiation for Stage III Non-Small Cell Lung Cancer: Randomized Phase III Trial RTOG 9410. JNCI J Natl Cancer Inst 2011;103(19):1452–60.

19. Albain KS, Swann RS, Rusch VW, et al. Radiotherapy plus chemotherapy with or without surgical resection for stage III non-small-cell lung cancer: a phase III randomised controlled trial. Lancet 2009;374(9687):379–86.

20. Fournel P, Robinet G, Thomas P, et al. Randomized Phase III Trial of Sequential Chemoradiotherapy Compared With Concurrent Chemoradiotherapy in Locally Advanced Non?Small-Cell Lung Cancer: Groupe Lyon-Saint-Etienne d'Oncologie Thoracique?Groupe Fran?ais de Pneumo-Canc?rologie NPC 95-01 Study. J Clin Oncol 2005;23(25):5910–7.

21. Charlson M, Szatrowski TP, Peterson J, Gold J. Validation of a combined comorbidity index. J Clin Epidemiol 1994;47(11):1245–51.

22. Firat S, Byhardt RW, Gore E. Comorbidity and Karnofksy performance score are independent prognostic factors in stage III non-small-cell lung cancer: an institutional analysis of patients treated on four RTOG studies. Radiation Therapy Oncology Group. Int J Radiat Oncol Biol Phys 2002;54(2):357–64.

23. Douillard J-Y, Rosell R, De Lena M, et al. Adjuvant vinorelbine plus cisplatin versus observation in patients with completely resected stage IB?IIIA non-small-cell lung cancer (Adjuvant Navelbine International Trialist Association [ANITA]): a randomised controlled trial. Lancet Oncol 2006;7(9):719–27.

24. Pisters KMW, Evans WK, Azzoli CG, et al. Cancer Care Ontario and American Society of Clinical Oncology Adjuvant Chemotherapy and Adjuvant Radiation Therapy for Stages I-IIIA Resectable Non?Small-Cell Lung Cancer Guideline. J Clin Oncol 2007;25(34):5506–18.

25. Baser S, Shannon VR, Eapen GA, et al. Smoking cessation after diagnosis of lung cancer is associated with a beneficial effect on performance status. Chest 2006;130(6):1784–90.

26. Temel JS, Blinderman CD, Jacobsen J, Pirl WF, Billings JA, Lynch TJ. Early Palliative Care for Patients with Metastatic Non–Small-Cell Lung Cancer. N Engl J Med 2010;363(19):733–42.

27. Burdett S, Stephens R, Stewart L, et al. Chemotherapy in Addition to Supportive Care Improves Survival in Advanced Non–Small-Cell Lung Cancer: A Systematic Review and Meta-Analysis of Individual Patient Data From 16 Randomized Controlled Trials. J Clin Oncol 2008;26(28):4617–25.

28. Pujol J-L, Barlesi F, Daur?s J-P. Should chemotherapy combinations for advanced non-small cell lung cancer be platinum-based? A meta-analysis of phase III randomized trials. Lung Cancer 2006;51(3):335–45.

29. Delbaldo C, Michiels S, Syz N, Soria J-C, Le Chevalier T, Pignon J-P. Benefits of Adding a Drug to a Single-Agent or a 2-Agent Chemotherapy Regimen in Advanced Non?Small-Cell Lung Cancer. JAMA 2004;292(4):470.

30. Rossi A, Chiodini P, Sun J-M, et al. Six versus fewer planned cycles of first-line platinum-based chemotherapy for non-small-cell lung cancer: a systematic review and meta-analysis of individual patient data. Lancet Oncol 2014;15(11):1254–62.

31. Gridelli C, Ardizzoni A, Le Chevalier T, et al. Treatment of advanced non-small-cell lung cancer patients with ECOG performance status 2: results of an European Experts Panel. Ann Oncol Off J Eur Soc Med Oncol 2004;15(3):419–26.

32. WHO. The World Health Organization histological typing of lung tumours. Second edition. Am J Clin Pathol 1982;77(2):123–36.

33. Gibbs AR, Thunnissen FB. Histological typing of lung and pleural tumours: third edition. J Clin Pathol 2001;54(7):498–9.

34. WHO. Pathology and genetics of tumours of the lung, pleura, thymus and heart. Lyon: 2004.

35. Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization Classification of Lung Tumors. J Thorac Oncol 2015;10(9):1243–60.

36. Travis WD, Brambilla E, Noguchi M, et al. International Association for the Study of Lung Cancer / American Thoracic Society / European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma. J Thorac Oncol 2011;6(2):244–85.

37. Travis WD, Brambilla E, Riely GJ. New pathologic classification of lung cancer: Relevance for clinical practice and clinical trials. J Clin Oncol 2013;31(8):992–1001.

38. Chan BA, Hughes BGM. Targeted therapy for non-small cell lung cancer: current standards and the promise of the future. Transl lung cancer Res 2015;4(1):36–54.

39. Li M, Zhang Q, Fu P, et al. Pemetrexed plus platinum as the first-line treatment option for advanced non-small cell lung cancer: a meta-analysis of randomized controlled trials. PLoS One 2012;7(5):e37229.

40. Scagliotti G, Hanna N, Fossella F, et al. The Differential Efficacy of Pemetrexed According to NSCLC Histology: A Review of Two Phase III Studies. Oncologist 2009;14(3):253–63.

41. Paz-Ares LG, de Marinis F, Dediu M, et al. PARAMOUNT: Final Overall Survival Results of the Phase III Study of Maintenance Pemetrexed Versus Placebo Immediately After Induction Treatment With Pemetrexed Plus Cisplatin for Advanced Nonsquamous Non–Small-Cell Lung Cancer. J Clin Oncol 2013;31(23):2895–902.

42. Soria J-C, Mauguen A, Reck M, et al. Systematic review and meta-analysis of randomised, phase II/III trials adding bevacizumab to platinum-based chemotherapy as first-line treatment in patients with advanced non-small-cell lung cancer. Ann Oncol 2013;24(1):20–30.

43. Schiller JH, Harrington D, Belani CP, et al. Comparison of Four Chemotherapy Regimens for Advanced Non?Small-Cell Lung Cancer. N Engl J Med 2002;346(2):92–8.

44. Solis LM, Behrens C, Raso MG, et al. Histologic patterns and molecular characteristics of lung adenocarcinoma associated with clinical outcome. Cancer 2012;118(11):2889–99.

45. Warth A, Muley T, Meister M, et al. The Novel Histologic International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society Classification System of Lung Adenocarcinoma Is a Stage-Independent Predictor of Survival. J Clin Oncol 2012;30(13):1438–46.

46. Tsao M-S, Marguet S, Le Teuff G, et al. Subtype Classification of Lung Adenocarcinoma Predicts Benefit From Adjuvant Chemotherapy in Patients Undergoing Complete Resection. J Clin Oncol 2015;33(30):3439–46.

47. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. N Engl J Med 2001;344(11):783–92.

48. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and Safety of a Specific Inhibitor of the BCR-ABL Tyrosine Kinase in Chronic Myeloid Leukemia. N Engl J Med 2001;344(14):1031–7.

49. Chapman PB, Hauschild A, Robert C, et al. Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation. N Engl J Med 2011;364(26):2507–16.

50. Lynch TJ, Bell DW, Sordella R, et al. Activating Mutations in the Epidermal Growth Factor Receptor Underlying Responsiveness of Non–Small-Cell Lung Cancer to Gefitinib. N Engl J Med 2004;350(21):2129–39.

51. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012;13(3):239–46.

52. Mok TS, Wu Y-L, Thongprasert S, et al. Gefitinib or Carboplatin–Paclitaxel in Pulmonary Adenocarcinoma. N Engl J Med 2009;361(10):947–57.

53. Sequist L V., Yang JC-H, Yamamoto N, et al. Phase III Study of Afatinib or Cisplatin Plus Pemetrexed in Patients With Metastatic Lung Adenocarcinoma With *EGFR* Mutations. J Clin Oncol 2013;31(27):3327–34.

54. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4–ALK fusion gene in non-small-cell lung cancer. Nature 2007;448(7153):561–6.

55. Christensen JG, Zou HY, Arango ME, et al. Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. Mol Cancer Ther 2007;6(12):3314–22.

56. Shaw AT, Kim D-W, Nakagawa K, et al. Crizotinib versus Chemotherapy in Advanced *ALK* -Positive Lung Cancer. N Engl J Med 2013;368(25):2385–94.

57. Solomon BJ, Mok T, Kim D-W, et al. First-Line Crizotinib versus Chemotherapy in *ALK* -Positive Lung Cancer. N Engl J Med 2014;371(23):2167–77.

58. Peters S, Camidge DR, Shaw AT, et al. Alectinib versus Crizotinib in Untreated *ALK* -Positive Non–Small-Cell Lung Cancer. N Engl J Med 2017;NEJMoa1704795.

59. Soria J-C, Tan DSW, Chiari R, et al. First-line ceritinib versus platinum-based chemotherapy in advanced ALK -rearranged non-small-cell lung cancer (ASCEND-4): a randomised, open-label, phase 3 study. Lancet 2017;389(10072):917–29.

60. Shaw AT, Ou S-HI, Bang Y-J, et al. Crizotinib in *ROS1* -Rearranged Non–Small-Cell Lung Cancer. N Engl J Med 2014;371(21):1963–71.

61. Collisson EA, Campbell JD, Brooks AN, et al. Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014;511(7511):543–50.

62. Hammerman PS, Lawrence MS, Voet D, et al. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489(7417):519–25.

63. Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G. Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci 1987;84(18):6379–83.

64. Bottaro DP, Rubin JS, Faletto DL, et al. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 1991;251(4995):802–4.

65. Schmidt C, Bladt F, Goedecke S, et al. Scatter factor/hepatocyte growth factor is essential for liver development. Nature 1995;373(6516):699–702.

66. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature 1995;376(6543):768–71.

67. Uehara Y, Minowa O, Mori C, et al. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature 1995;373(6516):702–5.

68. Chmielowiec J, Borowiak M, Morkel M, et al. c-Met is essential for wound healing in the skin. J Cell Biol 2007;177(1):151–62.

69. Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997;276(5309):60–6.

70. Ponzetto C, Bardelli A, Zhen Z, et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell 1994;77(2):261–71.

71. Weidner KM, Di Cesare S, Sachs M, Brinkmann V, Behrens J, Birchmeier W. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature 1996;384(6605):173–6.

72. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol 2003;4(12):915–25.

73. Cooper CS, Park M, Blair DG, et al. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature 1984;311(5981):29–33.

74. Rong S, Segal S, Anver M, Resau JH, Vande Woude GF. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci U S A 1994;91(11):4731–5.

75. Ponzo MG, Lesurf R, Petkiewicz S, et al. Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. Proc Natl Acad Sci 2009;106(31):12903–8.

76. Graveel C, Su Y, Koeman J, et al. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci 2004;101(49):17198–203.

77. Schaeper U, Vogel R, Chmielowiec J, Huelsken J, Rosario M, Birchmeier W. Distinct requirements for Gab1 in Met and EGF receptor signaling in vivo. Proc Natl Acad Sci 2007;104(39):15376–81.

78. Khoury H, Naujokas MA, Zuo D, et al. HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion. Mol Biol Cell 2005;16(2):550–61.

79. Bauer TW, Somcio RJ, Fan F, et al. Regulatory role of c-Met in insulin-like growth factor-I receptor-mediated migration and invasion of human pancreatic carcinoma cells. Mol Cancer Ther 2006;5(7):1676–82.

80. Yamamoto N, Mammadova G, Song RX-D, Fukami Y, Sato K -i. Tyrosine phosphorylation of p145met mediated by EGFR and Src is required for serum-independent survival of human bladder carcinoma cells. J Cell Sci 2006;119(22):4623–33.

81. Boon EMJ, van der Neut R, van de Wetering M, Clevers H, Pals ST. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. Cancer Res 2002;62(18):5126–8.

82. Zhang Y-W, Su Y, Volpert O V., Woude GF V. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. Proc Natl Acad Sci 2003;100(22):12718–23.

83. Ma PC, Tretiakova MS, MacKinnon AC, et al. Expression and mutational analysis of MET in human solid cancers. Genes Chromosom Cancer 2008;47(12):1025–37.

84. Network TCGAR. Comprehensive Molecular Characterization of Papillary Renal-Cell Carcinoma. N Engl J Med 2016;374(2):135–45.

85. Kuniyasu H, Yasui W, Kitadai Y, Yokozaki H, Ito H, Tahara E. Frequent amplification of the c-met gene in scirrhous type stomach cancer. Biochem Biophys Res Commun 1992;189(1):227–32.

86. Zeng Z-S, Weiser MR, Kuntz E, et al. c-Met gene amplification is associated with advanced stage colorectal cancer and liver metastases. Cancer Lett 2008;265(2):258–69.

87. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET Amplification Leads to Gefitinib Resistance in Lung Cancer by Activating ERBB3 Signaling. Science (80-) 2007;316(5827):1039–43.

88. Lutterbach B, Zeng Q, Davis LJ, et al. Lung Cancer Cell Lines Harboring MET Gene Amplification Are Dependent on Met for Growth and Survival. Cancer Res 2007;67(5):2081–8.

89. Harvey P, Warn A, Newman P, Perry LJ, Ball RY, Warn RM. Immunoreactivity for hepatocyte growth factor/scatter factor and its receptor, met, in human lung carcinomas and malignant mesotheliomas. J Pathol 1996;180(4):389–94.

90. Olivero M, Rizzo M, Madeddu R, et al. Overexpression and activation of hepatocyte growth factor/scatter factor in human non-small-cell lung carcinomas. Br J Cancer 1996;74(12):1862–8.

91. Ichimura E, Maeshima A, Nakajima T, Nakamura T. Expression of c-met/HGF receptor in human non-small cell lung carcinomas in vitro and in vivo and its prognostic significance. Jpn J Cancer Res 1996;87(10):1063–9.

92. Masuya D, Huang C, Liu D, et al. The tumour?stromal interaction between intratumoral c-Met and stromal hepatocyte growth factor associated with tumour growth and prognosis in non-small-cell lung cancer patients. Br J Cancer 2004;90(8):1555–62.

93. Okuda K, Sasaki H, Yukiue H, Yano M, Fujii Y. Met gene copy number predicts the prognosis for completely resected non-small cell lung cancer. Cancer Sci 2008;99(11):2280–5.

94. Go H, Jeon YK, Park HJ, Sung S-W, Seo J-W, Chung DH. High MET gene copy number leads to shorter survival in patients with non-small cell lung cancer. J Thorac Oncol 2010;5(3):305–13.

95. Tanaka A, Sueoka-Aragane N, Nakamura T, et al. Co-existence of positive MET FISH status with EGFR mutations signifies poor prognosis in lung adenocarcinoma patients. Lung Cancer 2012;75(1):89–94.

96. Park S, Choi Y-L, Sung CO, et al. High MET copy number and MET overexpression: poor outcome in non-small cell lung cancer patients. Histol Histopathol 2012;27(2):197–207.

97. Cappuzzo F, Marchetti A, Skokan M, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol 2009;27(10):1667–74.

98. Beau-Faller M, Ruppert A-M, Voegeli A-C, et al. MET Gene Copy Number in Non-small Cell Lung Cancer: Molecular Analysis in a Targeted Tyrosine Kinase Inhibitor Naïve Cohort. J Thorac Oncol 2008;3(4):331–9.

99. Dimou A, Non L, Chae YK, Tester WJ, Syrigos KN. MET Gene Copy Number Predicts Worse Overall Survival in Patients with Non-Small Cell Lung Cancer (NSCLC); A Systematic Review and Meta-Analysis. PLoS One 2014;9(9):e107677.

100. Guo B, Cen H, Tan X, Liu W, Ke Q. Prognostic value of MET gene copy number and protein expression in patients with surgically resected non-small cell lung cancer: a meta-analysis of published literatures. PLoS One 2014;9(6):e99399.

101. Bean J, Brennan C, Shih J-Y, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci U S A 2007;104(52):20932–7.

102. Wolff AC, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. J Clin Oncol 2006;25(1):118–45.

103. Varella-Garcia M. Stratification of non-small cell lung cancer patients for therapy with epidermal growth factor receptor inhibitors: the EGFR fluorescence in situ hybridization assay. Diagn Pathol 2006;1:19.

104. Weingertner N, Meyer N, Voegeli A-C, et al. Correlation between MET protein expression and MET gene copy number in a Caucasian cohort of non-small cell lung cancers according to the new IASLC/ATS/ERS classification. Pathology 2015;47(June):1.

105. Tran TN, Selinger CI, Kohonen-Corish MRJ, et al. Alterations of MET Gene Copy Number and Protein Expression in Primary Non-Small-Cell Lung Cancer and Corresponding Nodal Metastases. Clin Lung Cancer 2016;17(1):30–8.

106. Tachibana K, Minami Y, Shiba-Ishii A, et al. Abnormality of the hepatocyte growth factor/MET pathway in pulmonary adenocarcinogenesis. Lung Cancer 2012;75(2):181–8.

107. Onozato R, Kosaka T, Kuwano H, Sekido Y, Yatabe Y, Mitsudomi T. Activation of MET by Gene Amplification or by Splice Mutations Deleting the Juxtamembrane Domain in Primary Resected Lung Cancers. J Thorac Oncol 2009;4(1):5–11.

108. Zaffaroni D, Spinola M, Galvan A, et al. Met proto-oncogene juxtamembrane rare variations in mouse and humans: differential effects of Arg and Cys alleles on mouse lung tumorigenesis. Oncogene 2005;24(6):1084–90.

109. Kong-Beltran M, Seshagiri S, Zha J, et al. Somatic Mutations Lead to an Oncogenic Deletion of Met in Lung Cancer. Cancer Res 2006;66(1):283–9.

110. Frampton GM, Ali SM, Rosenzweig M, et al. Activation of MET via diverse exon 14 splicing alterations occurs in multiple tumor types and confers clinical sensitivity to MET inhibitors. Cancer Discov 2015;5(8):850–60.

111. Zou HY, Li Q, Lee JH, et al. An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. Cancer Res 2007;67(9):4408–17.

112. Camidge DR, Ou SHI, Shapiro G, et al. Efficacy and safety of crizotinib in patients with advanced c-MET-amplified non-small cell lung cancer (NSCLC). J Clin Oncol Oncol 2014;32(15).

113. Paik PK, Drilon A, Fan PD, et al. Response to MET inhibitors in patients with stage IV lung adenocarcinomas harboring met mutations causing exon 14 skipping. Cancer Discov 2015;5(8):842–50.

114. Awad M, Leonardi G, Kravets S, Dahlberg S. Impact of MET inhibitors on survival among patients (pts) with MET exon 14 mutant (METdel14) non-small cell lung cancer (NSCLC). [Internet]. 2017 [cited 2017 Jun 29];Available from: http://ascopubs.org/doi/abs/10.1200/JCO.2017.35.15_suppl.8511

115. Ou S-HI, Kwak EL, Siwak-Tapp C, et al. Activity of Crizotinib (PF02341066), a Dual Mesenchymal-Epithelial Transition (MET) and Anaplastic Lymphoma Kinase (ALK) Inhibitor, in a Non-small Cell Lung Cancer Patient with De Novo MET

Amplification. J Thorac Oncol 2011;6(5):942–6.

116. Caparica R, Yen CT, Coudry R, et al. Responses to Crizotinib Can Occur in High-Level MET-Amplified Non–Small Cell Lung Cancer Independent of MET Exon 14 Alterations. J Thorac Oncol 2017;12(1):141–4.

117. Goldman JW, Laux I, Chai F, et al. Phase 1 dose-escalation trial evaluating the combination of the selective MET (mesenchymal-epithelial transition factor) inhibitor tivantinib (ARQ 197) plus erlotinib. Cancer 2012;118(23):5903–11.

118. Sequist L V., Von Pawel J, Garmey EG, et al. Randomized phase II study of erlotinib plus tivantinib versus erlotinib plus placebo in previously treated non-small-cell lung cancer. J Clin Oncol 2011;29(24):3307–15.

119. Yoshioka H, Azuma K, Yamamoto N, et al. A randomized, double-blind, placebo-controlled, phase III trial of erlotinib with or without a c-Met inhibitor tivantinib (ARQ 197) in Asian patients with previously treated stage IIIB/IV nonsquamous nonsmall-cell lung cancer harboring wild-type epidermal growth factor receptor (ATTENTION study). Ann Oncol 2015;26(10):2066–72.

120. Scagliotti G, Von Pawel J, Novello S, et al. Phase III multinational, randomized, double-blind, placebo-controlled study of tivantinib (ARQ 197) plus erlotinib versus erlotinib alone in previously treated patients with locally advanced or metastatic nonsquamous non-small-cell lung cancer. J Clin Oncol 2015;33(24):2667–74.

121. Calles A, Kwiatkowski N, Cammarata BK, Ercan D, Gray NS, J??nne PA. Tivantinib (ARQ 197) efficacy is independent of MET inhibition in non-small-cell lung cancer cell lines. Mol Oncol 2015;9(1):260–9.

122. Merchant M, Ma X, Maun HR, et al. Monovalent antibody design and mechanism of action of onartuzumab, a MET antagonist with anti-tumor activity as a therapeutic agent. Proc Natl Acad Sci U S A 2013;110(32):E2987-96.

123. Spigel DR, Ervin TJ, Ramlau RA, et al. Randomized phase II trial of Onartuzumab in combination with erlotinib in patients with advanced non-small-cell lung cancer. J Clin Oncol 2013;31(32):4105–14.

124. Spigel DR, Edelman MJ, O'Byrne K, et al. Results From the Phase III Randomized Trial of Onartuzumab Plus Erlotinib Versus Erlotinib in Previously Treated Stage IIIB or IV Non–Small-Cell Lung Cancer: METLung. J Clin Oncol 2017;35(4):412–20.

125. Koeppen H, Yu W, Zha J, et al. Biomarker analyses from a placebo-controlled phase II study evaluating erlotinib??onartuzumab in advanced non-small cell lung cancer: MET expression levels are predictive of patient benefit. Clin Cancer Res 2014;20(17):4488–98.

126. Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. Science (80-) 2011;331(6024):1565–70.

127. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell 2011;144(5):646–74.

128. Waldmann H. Mechanisms of immunological tolerance. Clin Biochem 2016;49(4–5):324–8.

129. Taube JM, Anders RA, Young GD, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 2012;4(127):127ra37.

130. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002;8(8):793–800.

131. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012;366(26):2443–54.

132. Brahmer JR, Tykodi SS, Chow LQM, et al. Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. N Engl J Med 2012;366(26):2455–65.

133. Robert C, Schachter J, Long G V., et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. N Engl J Med 2015;372(26):2521–32.

134. Motzer RJ, Escudier B, McDermott DF, et al. Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. N Engl J Med 2015;373(19):1803–13.

135. Bellmunt J, de Wit R, Vaughn DJ, et al. Pembrolizumab as Second-Line Therapy for Advanced Urothelial Carcinoma. N Engl J Med 2017;376(11):1015–26.

136. Agata Y, Kawasaki A, Nishimura H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 1996;8(5):765–72.

137. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. Nat Rev Immunol 2008;8(6):467–77.

138. Taube JM, Klein A, Brahmer JR, et al. Association of PD-1, PD-1 Ligands, and Other Features of the Tumor Immune

Microenvironment with Response to Anti-PD-1 Therapy. Clin Cancer Res 2014;20(19):5064–74.

139. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515(7528):568–71.

140. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. N Engl J Med 2012;366(26):2443–2454.

141. Herbst RS, Soria J-C, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014;515(7528):563–7.

142. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Pembrolizumab versus Chemotherapy for PD-L1–Positive Non–Small-Cell Lung Cancer. N Engl J Med 2016;375(19):1823–33.

143. Carbone DP, Reck M, Paz-Ares L, et al. First-Line Nivolumab in Stage IV or Recurrent Non–Small-Cell Lung Cancer. N Engl J Med 2017;376(25):2415–26.

144. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non–Small-Cell Lung Cancer. N Engl J Med 2015;373(17):1627–39.

145. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non–Small-Cell Lung Cancer. N Engl J Med 2015;373(2):123–35.

146. Herbst RS, Baas P, Kim D-WW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): A randomised controlled trial. Lancet 2016;387(10027):1540–50.

147. Topalian SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nat Rev Cancer 2016;16(5):275–87.

148. Heppner G. Tumor heterogeneity. Cancer Res 1984;44(6):2259–65.

149. Nowell PC. The Clonal Evolution of Tumor Cell Populations. Sci New Ser 1950;194(4260):23–8.

150. McGranahan N, Swanton C, Srinivas R, et al. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. Cell 2017;168(4):613–28.

151. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. N Engl J Med 2012;366(10):883–92.

152. Bashashati A, Ha G, Tone A, et al. Distinct evolutionary trajectories of primary high-grade serous ovarian cancers revealed through spatial mutational profiling. J Pathol 2013;231(1):21–34.

153. Eckert MA, Pan S, Hernandez KM, et al. Genomics of Ovarian Cancer Progression Reveals Diverse Metastatic Trajectories Including Intraepithelial Metastasis to the Fallopian Tube. Cancer Discov 2016;6(12):1342–51.

154. Hao J-J, Lin D-C, Dinh HQ, et al. Spatial intratumoral heterogeneity and temporal clonal evolution in esophageal squamous cell carcinoma. Nat Genet 2016;48(12):1500–7.

155. Harbst K, Lauss M, Cirenajwis H, et al. Multiregion Whole-Exome Sequencing Uncovers the Genetic Evolution and Mutational Heterogeneity of Early-Stage Metastatic Melanoma. Cancer Res 2016;76(16):4765–74.

156. de Bruin EC, McGranahan N, Mitter R, et al. Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. Science (80-) 2014;346(6206):251–6.

157. Zhang J, Fujimoto J, Zhang J, et al. Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. Science 2014;346(6206):256–9.

158. Jamal-Hanjani M, Hackshaw A, Ngai Y, et al. Tracking Genomic Cancer Evolution for Precision Medicine: The Lung TRACERx Study. PLoS Biol 2014;12(7):1–7.

159. Jamal-Hanjani M, Wilson GA, McGranahan N, et al. Tracking the Evolution of Non–Small-Cell Lung Cancer. N Engl J Med 2017;NEJMoa1616288.

160. Kononen J, Bubendorf L, Kallioniemi a, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4(7):844–7.

161. Koeppen H, Januario T, Filvaroff E et al. Characterization and clinical validation of an immunohistochemical assay for Met in non-small cell lung cancer. In: 101st Annual Meeting of the United States and Canadian Academy of Pathology. Vancouver, British Columbia, Canada: 2012.

162. Lapère C, Cortot AB, Grégoire V, Cockenpot V, Tulasne D, Copin M-C. Preferential Localization of MET Expression at the

Invasion Front and in Spreading Cells Through Air Spaces in Non–Small Cell Lung Carcinomas. Am J Surg Pathol 2017;41(3):414–22.

163. Schildhaus HU, Schultheis AM, Rüuschoff J, et al. Met amplification status in therapy-naïve adeno- And squamous cell carcinomas of the lung. Clin Cancer Res 2015;21(4):907–15.

164. Noonan SA, Berry L, Lu X, et al. Identifying the appropriate FISH criteria for defining MET copy number-driven lung adenocarcinoma through oncogene overlap analysis. J Thorac Oncol 2016;11(8):1293–304.

165. McLaughlin J, Han G, Schalper KA, et al. Quantitative Assessment of the Heterogeneity of PD-L1 Expression in Non-Small-Cell Lung Cancer. JAMA Oncol 2015;2(1):1–9.

166. Rehman JA, Han G, Carvajal-Hausdorf DE, et al. Quantitative and pathologist-read comparison of the heterogeneity of programmed death-ligand 1 (PD-L1) expression in non-small cell lung cancer. Mod Pathol 2016;1–10.

167. Gniadek TJ, Li QK, Tully E, Chatterjee S, Nimmagadda S, Gabrielson E. Heterogeneous expression of PD-L1 in pulmonary squamous cell carcinoma and adenocarcinoma: implications for assessment by small biopsy. Mod Pathol 2017;1–9.

168. Ilie M, Long-Mira E, Bence C, et al. Comparative study of the PD-L1 status between surgically resected specimens and matched biopsies of NSCLC patients reveal major discordances: A potential issue for anti-PD-L1 therapeutic strategies. Ann Oncol 2016;27(1):147–53.

169. Leora Horn, David R. Spigel, Scott N. Gettinger, Scott Joseph Antonia, Michael S. Gordon, Roy S. Herbst, Lecia V. Sequist, Colombe Chappey, Marcin Kowanetz, Alan Sandler J-CS. Clinical activity, safety and predictive biomarkers of the engineered antibody MPDL3280A (anti-PDL1) in non-small cell lung cancer (NSCLC): update from a phase Ia study. In: 2015 ASCO Annual Meeting. 2015. p. suppl; abstr 8029.

170. Li C, Huang C, Mok TS, et al. Comparison of 22C3-PD-L1 expression between surgically resected specimens and paired tissue microarrays in non-small cell lung cancer. J Thorac Oncol 2017;

171. Mansfield AS, Murphy SJ, Peikert T, et al. Heterogeneity of Programmed Cell Death Ligand 1 Expression in Multifocal Lung Cancer. Clin Cancer Res 2016;22(9):2177–82.

172. Uruga H, Bozkurtlar E, Huynh TG, et al. Programmed Cell Death Ligand (PD-L1) Expression in Stage II and III Lung Adenocarcinomas and Nodal Metastases. J Thorac Oncol 2016;

173. Pinato DJ, Shiner RJ, White SDT, et al. Intra-tumoral heterogeneity in the expression of programmed-death (PD) ligands in isogeneic primary and metastatic lung cancer: Implications for immunotherapy. Oncoimmunology 2016;5(9):e1213934.

174. Mansfield AS, Aubry MC, Moser JC, et al. Temporal and spatial discordance of programmed cell death-ligand 1 expression and lymphocyte tumor infiltration between paired primary lesions and brain metastases in lung cancer. J Natl Cancer Inst Prostate Br J Cancer Curr Opin Pharmacol Ann Oncol 2000;92(27):205–16.

175. Camidge D, Moran T, Demedts I, Grosch H. A randomized, open-label, phase 2 study of emibetuzumab plus erlotinib (LY+ E) and emibetuzumab monotherapy (LY) in patients with acquired resistance to erlotinib. 2016;

176. Wu Y, Kim D, Felip E, Zhang L, Liu X, Zhou C. efficacy results of a single-arm ph ib/II study of capmatinib (INC280)+ gefitinib in patients (pts) with EGFR-mutated (mut), cMET-positive (cMET+) non-small cell lung …. 2016;

177. Schuler M, Berardi R, Lim W, Geel R, Jonge M De. Phase (Ph) I study of the safety and efficacy of the cMET inhibitor capmatinib (INC280) in patients (pts) with advanced cMET+ non-small cell lung cancer (NSCLC). 2016;

178. Awad MM, Oxnard GR, Jackman DM, et al. MET exon 14 mutations in Non-small-cell lung cancer are associated with advanced age and stage-dependent MET genomic amplification and c-Met overexpression. J Clin Oncol 2016;34(7):721–30.

179. Tong JH, Yeung SF, Chan AWH, et al. MET Amplification and Exon 14 Splice Site Mutation Define Unique Molecular Subgroups of Non-Small Cell Lung Carcinoma with Poor Prognosis. Clin Cancer Res 2016;22(12):3048–56.

180. Watermann I, Schmitt B, Stellmacher F, et al. Improved diagnostics targeting c-MET in non-small cell lung cancer: expression, amplification and activation? Diagn Pathol 2015;10:130.

181. Tsuta K, Kozu Y, Mimae T, et al. C-MET/phospho-MET protein expression and MET gene copy number in non-small cell lung carcinomas. J Thorac Oncol 2012;7(2):331–9.

182. Jurmeister P, Lenze D, Berg E, et al. Parallel screening for ALK, MET and ROS1 alterations in non-small cell lung cancer with implications for daily routine testing. Lung Cancer 2015;87(2):122–9.

183. Ikeda S, Okamoto T, Okano S, et al. PD-L1 is upregulated by simultaneous amplification of the PD-L1 and JAK2 genes in non-small cell lung cancer. J Thorac Oncol 2016;11(1):62–71.

184. Budczies J, Bockmayr M, Denkert C, et al. Pan-cancer analysis of copy number changes in programmed death-ligand 1 (PD-L1, PDL1) – associations with gene expression, mutational load, and survival. Genes Chromosom Cancer 2016;55(8):626–39.

185. Goldmann T, Kugler C, Reinmuth N, Vollmer E, Reck M. PD-L1 copy number gain in nonsmall-cell lung cancer defines a new subset of patients for anti PD-L1 therapy. Ann Oncol 2016;27(1):206–7.

186. Inoue Y, Yoshimura K, Mori K, et al. Clinical significance of PD-L1 and PD-L2 copy number gains in non-small-cell lung cancer. Oncotarget 2016;7(22):32113–28.

187. Death C, Pd-l L, Small N, et al. Comparison of Different Antibody Clones for Immunohistochemistry Detection of Programmed. 2017;0(0):1–11.

188. Rimm DL, Han G, Taube JM, et al. A Prospective, Multi-institutional, Pathologist-Based Assessment of 4 Immunohistochemistry Assays for PD-L1 Expression in Non–Small Cell Lung Cancer. JAMA Oncol 2017;6520:1–8.

189. Mahoney KM, Sun H, Liao X, et al. PD-L1 Antibodies to Its Cytoplasmic Domain Most Clearly Delineate Cell Membranes in Immunohistochemical Staining of Tumor Cells. Cancer Immunol Res 2015;3(12):1308–15.

190. Cogswell J, Inzunza HD, Wu Q, et al. An Analytical Comparison of Dako 28-8 PharmDx Assay and an E1L3N Laboratory-Developed Test in the Immunohistochemical Detection of Programmed Death-Ligand 1. Mol Diagnosis Ther 2017;21(1):85–93.

191. Ratcliffe MJ, Sharpe A, Midha A, et al. Agreement between Programmed Cell Death Ligand-1 Diagnostic Assays across Multiple Protein Expression Cutoffs in Non–Small Cell Lung Cancer. Clin Cancer Res 2017;clincanres.2375.2016.

192. Brunnström H, Johansson A, Westbom-Fremer S, et al. PD-L1 immunohistochemistry in clinical diagnostics of lung cancer: inter-pathologist variability is higher than assay variability. Nat Publ Gr 2017;1–11.

193. Smith J, Robida MD, Acosta K, et al. Quantitative and qualitative characterization of Two PD-L1 clones: SP263 and E1L3N. Diagn Pathol 2016;11(1):44.

194. Gaule P, Smithy JW, Toki M, et al. A Quantitative Comparison of Antibodies to Programmed Cell Death 1 Ligand 1. JAMA Oncol 2017;3(2):256.

195. Scheel AH, Dietel M, Heukamp LC, et al. Harmonized PD-L1 immunohistochemistry for pulmonary squamous-cell and adenocarcinomas. Mod Pathol 2016;1–8.

196. Hirsch FR, McElhinny A, Stanforth D, et al. PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. J Thorac Oncol 2017;12(2):208–22.

197. Lerner A, Yarmus L, Feller-Kopman D, Lee H. PD-L1 assessment in FNA (EBUS) dervied samples. 2017;

198. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene 2010;29(8):1093–1102.

199. Pagès F, Berger A, Camus M, et al. Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer. N Engl J Med 2005;353(25):2654–66.

200. Teng MWL, Ngiow SF, Ribas A, Smyth MJ. Classifying cancers basedon T-cell infiltration and PD-L1. Cancer Res 2015;75(11):2139–45.

201. Donnem T, Hald SM, Paulsen E-E, et al. Stromal CD8+ T-cell Density--A Promising Supplement to TNM Staging in Non-Small Cell Lung Cancer. Clin Cancer Res 2015;21(11):2635–43.

202. Donnem T, Kilvaer TK, Andersen S, et al. Strategies for clinical implementation of TNM-Immunoscore in resected nonsmall-cell lung cancer. Ann Oncol 2016;27(2):225–32.

203. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. Clin Cancer Res 2008;14(16):5220–7.

204. Brambilla E, Le Teuff GG, Marguet S, et al. Prognostic effect of tumor lymphocytic infiltration in resectable non-small-cell lung cancer. J Clin Oncol 2016;34(11):1223–30.

205. Obeid JM, Wages NA, Hu Y, Deacon DH, Slingluff CL. Heterogeneity of CD8+ tumor-infiltrating lymphocytes in non-small-cell lung cancer: impact on??patient prognostic assessments and comparison of quantification by different sampling strategies. Cancer Immunol Immunother 2017;66(1):33–43.

8. APPENDIX I. Complementary Tables

Table 1. Studies analyzing MET gene and protein expression in NSCLC

Author	Year	N	Histology	*MET* analysis	*MET* criteria	*MET*+ (%)	IHC criteria*	IHC+ (%)
Cappuzzo[1]	2008	166	NSCLC	FISH	Cappuzzo	7.8	-	-
Okuda[2]	2008	213	NSCLC	qPCR	>3 MET copies	5.6	Strong (2+) staining	38.8[a]
Cappuzzo[3]	2009	435	NSCLC	FISH	Cappuzzo	11.1	-	-
Kubo[4]	2009	100	NSCLC	qPCR	>5 MET copies	2	-	-
Onozato[5]	2009	187	NSCLC	qPCR	≥2 MET copies	1.1	-	-
Camidge[6]	2010	61	NSCLC	FISH	UCCC	0	-	-
Go[7]	2010	180	NSCLC	FISH	UCCC / Cappuzzo	16.7 / 6.7	-	-
Onitsuka[8]	2010	183	ADC	qPCR	>1.31 MET copies	4	-	-
Gumustekin[9]	2011	63	NSCLC	-	-	-	Intensity(0-3) x %(0-4)	81
Chen[10]	2011	208	NSCLC	qPCR	≥3 MET copies	10.6	-	-
Tsuta[11]	2012	906	NSCLC	BISH	UCCC	10.9	≥10% positive cells	22.2
Dziadziuszko[12]	2012	174	NSCLC	SISH	Cappuzzo	10	Hscore median / Metmab	25
Park[13]	2012	380	NSCLC	FISH	UCCC / Cappuzzo	11.1 / 7.1	Intensity(0-3) x %(0-4)	13.7
Tachibana[14]	2012	106	ADC	FISH	UCCC	10.4	≥10% 3+ cells or ≥40% 2+ cells	28.3
Tanaka[15]	2012	138	ADC	FISH	Cappuzzo / *MET*/CEP7 ratio ≥2	15 / 4.0	-	-
Jin[16]	2013	141	ADC[b]	SISH	≥3.4 MET copies/cell	24.1	H-score median	50
Sun[17]	2013	61	NSCLC	qPCR	≥3 MET copies	18	Intensity (0-3) x % (0-4)	59
Jurmeister[18]	2014	473	NSCLC	FISH	UCCC / Cappuzzo *MET*/CEP7 ratio ≥2	10.2 / 3.9 1.4	H-score median / Metmab	17
Schildhaus[19]	2014	693	NSCLC	FISH	mUCCC	33	Metmab	27.7
Li[20]	2015	90	NSCLC[c]	-	-	-	H-score median	50
Park[21]	2015	316	ADC	FISH	UCCC	38.9	Metmab	24.4
Weingertner[22]	2015	201	NSCLC	SISH	Cappuzzo / *MET*/CEP7 ratio ≥2	14 / 7.0	Metmab / ≥10% 3+ cells / H-score	44 / 28 / 42

Table 1. (cont.)

Author	Year	N	Histology	*MET* analysis	*MET* criteria	*MET*+ (%)	IHC criteria	IHC+ (%)
Tran[23]	2015	300	NSCLC	FISH	UCCC	8.1	Metmab	10.3
Watermann[24]	2015	222	NSCLC	FISH	mUCCC(2)	3.7	Metmab	21.5
Wang[25]	2015	117	NSCLC	-	-	-	H-score	69.2
Gao[26]	2016	198	NSCLC	-	-	-	≥50% 3+ cells	29.3
Noonan[27]	2016	1164	NSCLC	FISH	Cappuzzo / Camidge	14 / 0.3	-	-
Tong[28]	2016	687	NSCLC	FISH	Cappuzzo / Camidge	2.3 / 1.2	Metmab	33.5
Awad[29]	2016	933	NSCLC	NGS	*MET*/CEP7 > 3:1	0.6	-	-
Lapère[30]	2017	64	NSCLC	SISH	mUCCC	3.1	H-score median / Metmab	34

Abbreviations: *NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; qPCR, quantitative polymerase chain reaction; FISH, fluorescence insitu hybridization; SISH, silver in situ hybridization; BISH, bright-field insitu hybridization; IHC, immunohistochemistry; UCCC, University of Colorado Cancer Center; mUCCC modified UCCC; NGS, next-generation sequencing; MET,*

* All the studies used the c-MET SP44 Rabbit clone antibody (Ventana Medical Systems, Tucson, AZ) except for Gumustekin et al and Sun et al (Santa Cruz Laboratories), Okuda et al (Spring Bioscience), Park (Zymed Labs), Tachibana et al (IBL Labs), and Wang et al (Cell Signaling).

[a]Only evaluated in 23% of the study population

[b]Only patients with stage I disease

[c]Only patients with advanced disease

Table 2. Discordance between MET IHC and FISH

Author	Year	FISH criteria	IHC criteria	IHC+/FISH-	IHC-/FISH+
Okuda[2]	2008	>3 *MET* copies	Strong (2+) staining	30.8%	30%
Tsuta[11]	2012	UCCC	≥10% positive cells	71.2%	5.8%
Park[13]	2012	UCCC	Intensity (0-3) x % (0-4)	69.2%	7.9%
Park[13]	2012	Cappuzzo	Intensity (0-3) x % (0-4)	76.9%	4.6%
Tachibana[14]	2012	UCCC	≥10% 3+ cells or ≥40% 2+ cells	23.2%	30%
Jurmeister[18]	2014	UCCC	Metmab	9.4%	15.6%
Jurmeister[18]	2014	Cappuzzo	Metmab	14.2%	11.8%
Jurmeister[18]	2014	Pathvysion	Metmab	18.1%	0%
Schildhaus[18]	2014	mUCCC	Metmab	72.2%	0%
Park[21]	2015	UCCC	Metmab	42.8%	32.2%
Weingertner[22]	2015	Cappuzzo	Metmab	68.5%	0.9%
Weingertner[22]	2015	MET/CEP7 ≥2	Metmab	86.5%	1.8%
Tong[28]	2016	Cappuzzo / Camidge / MET/CEP7 ≥2	Metmab	29.8%	0.6%

Abbreviations: *FISH, fluorescence in situ hybridization; UCCC, University of Colorado Cancer Center; IHC, immunohistochemistry; MET, MET Proto-Oncogene*

FISH and IHC positivity Criteria (Table 1 and Table 2): *Cappuzzo*[3]*:* mean MET gene copy number ≥ 5 ; *UCCC*[23]*:* MET/CEP7-ratio ≥2, small gene clusters (4-10 copies), or innumerable tight gene clusters in >10% of tumor cells, or larger and brighter MET signals than CEP7 signals in >10% of tumor cells, or >15 MET signals in >10% of tumor cells, or ≥4 MET signals in ≥40% of tumor cells; *Camidge:* *MET*/CEP7 ratio ≥ 5. *H-score*[12]*:* the result of the product of staining intensity (0-4) x percentage of stained cells (0-100%), positivity cut-off usually set at the median. Metmab[31]: ≥50% of cells showing moderate (2+) or strong (3+) MET staining (SP144 antibody).

Table 3A. Efficacy of Nivolumab according to PD-L1 IHC

Author, Year	Phase	LOT	N	Histology	Agents	PD-L1 clone	PD-L1 (%N)	ORR (%)	PFS (mo)	OS (mo)
Topalian, 2012[32]	I	≥2nd	10	NSCLC	Nivolumab	28-8	≥5% (50)	20	NR	NR
							<5% (50)	0		
Gettinger, 2015[33]	I	≥2nd	68	NSCLC	Nivolumab	28-8	≥5% (48.5)	15	3.3	7.8
							<5% (51.5)	14	1.8	10.5
Gettinger, 2016[34]	I	1st	46	NSCLC	Nivolumab	28-8	≥50% (26.1)	50	8.3	83[I]
							≥25% (39.1)	44	5.8	78[I]
							≥10% (43.5)	40	5.2	80[I]
							≥5% (56.5)	31	3.5	73[I]
							≥1% (69.7)	28	3.5	69[I]
							<50% (73.9)	15	2.4	68[I]
							<25% (60.9)	11	2.4	68[I]
							<10% (56.5)	12	3.5	65[I]
							<5% (43.5)	15	5.0	70[I]
							<1% (30.4)	14	6.6	79[I]
Rizvi, 2015[35]	II	≥3rd	76	Squamous	Nivolumab	28-8	≥10% (32.9)	24	1.9	8.2
							≥5% (32.9)	24		
							≥1% (59.2)	20		
							<10% (67.1)	14		
							<5% (67.1)	14		
							<1% (40.8)	13		
Brahmer, 2015[36]	III	2nd	225	Squamous	Nivolumab	28-8	≥10% (27)	19	3.5	9.2
							≥5% (31)	21		
							≥1% (47)	17		
							<10% (60)	16		
							<5% (56)	15		
							<1% (40)	17		

Borghaei, 2015[37]	III	2nd	455	Nonsquam.	Docetaxel	28-8	≥10% (24)	9	2.8	6
							≥5% (29)	8		
							≥1% (41)	11		
							<10% (55)	11		
							<5% (50)	12		
							<1% (38)	10		
					Nivolumab	28-8	≥10% (37)	37	5	19.9
							≥5% (41)	36	5	19.4
							≥1% (53)	31	4.2	17.7
							<10% (63)	11	2.1	9.9
							<5% (59)	10	2.1	9.8
							<1% (47)	9	2.1	10.5
					Docetaxel	28-8	≥10% (35)	13	3.7	8
							≥5% (38)	13	3.8	8.1
							≥1% (55)	12	4.5	9
							<10% (65)	14	4.2	10.3
							<5% (62)	14	4.2	10.1
							<1% (45)	15	3.6	10.1
Carbone, 2017[38]	III	1st	423	NSCLC	Nivolumab	28-8	≥5% (100)	26.1	4.2	14.4
					PBD	28-8	≥5% (100)	33.5	5.9	13.2

Abbreviations: LOT, line of treatment; NSCLC, non-small cell lung cancer; PBD, platinum-based doublet; NReach, not reached; TC, tumor cells; IC, immune cells; NE, not estimable; NR, not reported

[l]OS expressed as % 1-year survival.

Table 3B. Efficacy of Pembrolizumab according to PD-L1 IHC

Author, Year	Phase	LOT	N	Histology	Agents	PD-L1 clone	PD-L1 (%N)	ORR (%)	PFS (mo)	OS (mo)
Garon, 2015[39]	I	Any	220	NSCLC	Pembrolizumab	22C3	≥50% (33.2)	45.2	6.4	NReach
							1-49% (46.8)	16.5	4.1	10.6
							<1% (12.7)	10.7	4	10.4
Hui, 2017[40]	I	1st	101	NSCLC	Pembrolizumab	22C3	≥50% (26.7)	52	12.5	NReach
							1-49% (51.4)	17	4.2	19.5
							<1% (11.9)	8	3.5	14.7
Herbst, 2016[41]	II/III	≥2nd	1034	NSCLC	Pembrolizumab 2mg/kg	22C3	≥50% (40)	30.2	5	14.9
							Total pop. (100)	18	3.9	10.4
					Pembrolizumab 10mg/kg	22C3	≥50% (44)	29.1	5.2	17.3
							Total pop. (100)	18.5	4	12.7
					Docetaxel	22C3	≥50% (44)	8	4.1	8.2
							Total pop. (100)	9.3	4	8.5
Reck, 2016[42]	III	1st	305	NSCLC	Pembrolizumab	22C3	≥50% (100)	44.8	10.3	NReach
					PBD	22C3	≥50% (100)	27.8	6	NReach

Abbreviations: LOT, line of treatment; NSCLC, non-small cell lung cancer; PBD, platinum-based doublet; NReach, not reached; TC, tumor cells; IC, immune cells; NE, not estimable; NR, not reported

Table 3C. Efficacy of Atezolizumab according to PD-L1 IHC

Author, Year	Phase	LOT	N	Histology	Agents	PD-L1 clone	PD-L1 (%N)	ORR (%)	PFS (mo)	OS (mo)
Herbst, 2014[I,43]	I	Any	53	NSCLC	Atezolizumab	SP142	IC3 (11.3)	83.3	NE	NR
							IC2 (13.2)	14.3	11	
							IC1 (24.5)	15.8	6	
							IC0 (37.7)	25	13	
							TC3 (15.1)	37.5	NR	
							TC2 (1.9)	0		
							TC1 (5.7)	33.3		
							TC0 (64.2)	23.5		
Fehrenbacher, 2016[II,44]	II	2nd/3rd	287	NSCLC	Atezolizumab	SP142	TC or IC 3 (16.4)	37.5	7.8	15.5
							TC or IC 2 (20.2)	7.7	2	9
							TC or IC 1 (31.4)	14	2.8	15.6
							TC and IC 0 (32.1)	7.8	1.7	9.7
					Docetaxel	SP142	TC or IC 3 (16.4)	13	3.9	11.1
							TC or IC 2 (20.2)	15.6	2.7	6.2
							TC or IC 1 (31.4)	19.1	3.5	12.4
							TC and IC 0 (32.1)	9.7	4.1	9.7
Rittmeyer, 2017[45]	III	2nd/3rd	850	NSCLC	Atezolizumab	SP142	TC or IC 3 (8.5)	30.6	4.2	13.8
							TC or IC 2/3 (15.2)	22.5	4.1	
							TC or IC 1/2/3 (28.4)	17.8	2.8	
							TC or IC 0 (21.2)	7.8	4	
					Docetaxel	SP142	TC or IC 3 (7.3)	10.8	3.3	9.6
							TC or IC 2/3 (16)	12.5	3.6	
							TC or IC 1/2/3 (26.1)	16.2	4.1	
							TC or IC 0 (23.4)	10.6	2.6	
Peters, 2017[III,46]	II	1st	142	NSCLC	Atezolizumab	SP142	TC or IC 3 (46.8)	20	5.6	26.9
							TC or IC 2 (53.2)	10	5.3	20.1

Abbreviations: LOT, line of treatment; NSCLC, non-small cell lung cancer; PBD, platinum-based doublet; NReach, not reached; TC, tumor cells; IC, immune cells; NE, not estimable; NR, not reported

[I]Specimens were scored as IHC 0, 1, 2, or 3 if <1%, ≥1% but <5%, ≥5% but <10%, or ≥10% of cells (IC or TC) per area were PD-L1 positive, respectively.

[II]Specimens were scored for as IC 1, 2 or 3 if <1%, ≥1% but <5%, ≥5% but <10%, or ≥10% of immune cells per area were PD-L1 positive, and TC 1, 2 or 3 if <1%, ≥1% but <5%, ≥5% but <50%, or ≥50% of tumor cells per area were PD-L1 positive. In this randomized study, the separate percentages of PD-L1 categories among the two treatment subroups are assumed the same in this table, but not reported specifically in the published material of the study.

[III]The study included three cohorts, divided by the number of previous treatment. This table only shows results for the previously untreated cohort of the trial.

Table 3D. Efficacy of Durvalumab and Avelumab according to PD-L1 IHC

Author, Year	Phase	LOT	N	Histology	Agents	PD-L1 clone	PD-L1 (%N)	ORR (%)	PFS (mo)	OS (mo)
Garassino, 2017[I,47]	I	Any	307	NSCLC	Durvalumab	SP263	≥90% (22.1)	30.9	2.4	NR
							≥25% (47.6)	16.4	3.3	10.9
							<25% (30.3)	7.5	1.9	9.3
Gulley, 2016[II,48]	I	≥2nd	142	NSCLC	Avelumab	73-10	≥25% (37)	17	11.9	8.4
							≥10% (19)	15	8.4	8.5
							≥5% (59)	14	11.9	10.6
							≥1% (86)	14	12	8.9
							<25% (63)	11	10.8	8.6
							<10% (81)	13	11.3	8.9
							<5% (51)	12	7.8	8.4
							<1% (14)	10	5.9	4.6

Abbreviations: LOT, line of treatment; NSCLC, non-small cell lung cancer; PBD, platinum-based doublet; NReach, not reached; TC, tumor cells; IC, immune cells; NE, not estimable; NR, not reported

[I]Only EGFR/ALK wild-type patients included.

[II]Survival times expressed in weeks

Supplementary Table 1. Additional content of Table 1.

Author	Year	Sample	IHC/FISH correlation	MET association with Clinical variables	MET assicuatuion with prognostic
Cappuzzo	2008	TMA	-	No	No
Okuda	2008	WTS	Yes (Chi-sq)	No	MET CNG with poor 5-yr OS (not sig. in MA)
Cappuzzo	2009	TMA	-	FISH+ with high stage and grade	FISH+ with poor OS (MA)
Kubo	2009	WTS	NA	-	-
Onozato	2009	WTS	NA	No amplifications in SCC detected	-
Camidge	2010	WTS	-	-	-
Go	2010	TMA	NA	MET+ with SCC histology	MET FISH+ with worse OS in patients with advanced stage and SCC histology (MA)
Onitsuka	2010	WTS	NA	No	-
Gumustekin	2011	WTS	NA	No	No
Chen	2011	WTS	NA	No	FISH+ with poor OS (not sig. in MA)
Tsuta	2012	TMA	Yes (Chi-sq)	MET IHC almost absent (0.7%) in SCC	BISH+ with poor OS in nsNSCLC (MA)
Dziadziuszko	2012	TMA	Yes (r=0.42)	No	No
Park	2012	TMA	Yes (Chi-sq)	MET IHC with young age, female gender, non-smoking history, ADC histology	MET IHC and FISH with worse DFS and OS (not sig. in MA)
Tachibana	2012	WTS	Yes (p=0.002)	MET IHC and FISH with pleural, vascular and lymphatic invasion, N+ and high grade	No
Tanaka	2012	WTS	NA	No	FISH+ (Pathvysion) with poor RFS and poor OS (no MA)
Jin	2013	TMA	No (r=0.13)	SISH+ with invasion, higher stage and inversely with lepidic	SISH+ with poor DFS (MA)
Sun	2013	WTS	Yes (r=0.39)	MET IHC+ with poor differentiation and MET FISH+ with N+ and advanced stage	MET IHC and FISH with worse OS (MA)
Jurmeister	2014	TMA	Yes (Chi-sq)	MET+ with lymphatic and pleural invasion and papillary growth pattern	No
Schildhaus	2014	WTS	Yes	No	-

Li	2015	WTS	-	No	No
Park	2015	TMA	Yes (Chi-sq)	MET FISH+ with young age, nodal involvement and advance stage	No
Weingertner	2015	TMA	Yes (Chi-sq)	MET IHC and FISH with ADC, high grade and sarcomatoid subtype	MET IHC+ predicts poor OS in non-smokers (MA)
Tran	2015	TMA	Yes (V=0.21)	MET IHC with female sex and ADC histology	MET IHC and FISH with better OS (MA)
Watermann	2015	TMA	No (r=0.06)	No	No
Wang	2015	TMA	NA	MET IHC with pN+	MET IHC with poor OS (MA)
Gao	2016	WTS	NA	MET associated with ADC histology	No
Noonan	2016	WTS	NA	MET+ with adrenal metastases	-
Tong	2016	TMA	Yes (Chi-sq)	MET IHC with adenocarcinoma, *MET* high-level amplification with sarcomatoid histology	*MET* high-level amplification with poor OS (MA), but not MET IHC
Awad	2016	Biopsies	-	-	-
Lapère	2017	WTS	No	MET IHC+ more frequent in ADC	-

Abbreviations: *TMA, tissue microarray; WTS, whole tissue section, IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; SISH, silver in situ hybridization; BISH, bright-field in situ hybridization; CNG, copy number gain; Chi-sq, Chi-square test; r, Spearman correlation test; ADC, adenocarcinoma; SCC, squamous cell carcinoma; OS, overall survival; DFS, disease-free survival; RFS, recurrence-free survival; MA, multivariate analysis*

Table References

1. Cappuzzo F, Jänne PA, Skokan M, et al. MET increased gene copy number and primary resistance to gefitinib therapy in non-small-cell lung cancer patients. Ann Oncol 2009;20(2):298–304.
2. Okuda K, Sasaki H, Yukiue H, Yano M, Fujii Y. Met gene copy number predicts the prognosis for completely resected non-small cell lung cancer. Cancer Sci 2008;99(11):2280–5.
3. Cappuzzo F, Marchetti A, Skokan M, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol 2009;27(10):1667–74.
4. Kubo T, Yamamoto H, Lockwood WW, et al. MET gene amplification or EGFR mutation activate MET in lung cancers untreated with EGFR tyrosine kinase inhibitors. Int J Cancer 2009;124(8):1778–84.
5. Onozato R, Kosaka T, Kuwano H, Sekido Y, Yatabe Y, Mitsudomi T. Activation of MET by Gene Amplification or by Splice Mutations Deleting the Juxtamembrane Domain in Primary Resected Lung Cancers. J Thorac Oncol 2009;4(1):5–11.
6. Camidge DR, Kono SA, Flacco A, et al. Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. Clin Cancer Res 2010;16(22):5581–90.
7. Go H, Jeon YK, Park HJ, Sung S-W, Seo J-W, Chung DH. High MET gene copy number leads to shorter survival in patients with non-small cell lung cancer. J Thorac Oncol 2010;5(3):305–13.
8. Onitsuka T, Uramoto H, Ono K, et al. Comprehensive molecular analyses of lung adenocarcinoma with regard to the epidermal growth factor receptor, K-ras, MET, and hepatocyte growth factor status. J Thorac Oncol 2010;5(5):591–6.
9. Gumustekin M, Kargi A, Bulut G, et al. HGF/c-Met overexpressions, but not met mutation, correlates with progression of non-small cell lung cancer. Pathol Oncol Res 2012;18(2):209–18.
10. Chen Y-TMS, Chang JW-CMD, Liu H-PMD, et al. Clinical Implications of High MET Gene Dosage in Non-Small Cell Lung Cancer Patients without Previous Tyrosine Kinase Inhibitor Treatment. J Thorac Oncol 2011;6(12):2027–35.
11. Tsuta K, Kozu Y, Mimae T, et al. C-MET/phospho-MET protein expression and MET gene copy number in non-small cell lung carcinomas. J Thorac Oncol 2012;7(2):331–9.
12. Dziadziuszko R, Wynes MW, Singh S, et al. Correlation between MET gene copy number by silver in situ hybridization and protein expression by immunohistochemistry in non-small cell lung cancer. J Thorac Oncol 2012;7(2):340–7.
13. Park S, Choi Y-L, Sung CO, et al. High MET copy number and MET overexpression: poor outcome in non-small cell lung cancer patients. Histol Histopathol 2012;27(2):197–207.
14. Tachibana K, Minami Y, Shiba-Ishii A, et al. Abnormality of the hepatocyte growth factor/MET pathway in pulmonary adenocarcinogenesis. Lung Cancer 2012;75(2):181–8.
15. Tanaka A, Sueoka-Aragane N, Nakamura T, et al. Co-existence of positive MET FISH status with EGFR mutations signifies poor prognosis in lung adenocarcinoma patients. Lung Cancer 2012;75(1):89–94.
16. Jin Y, Sun P-L, Kim H, et al. MET gene copy number gain is an independent poor prognostic marker in Korean stage I lung adenocarcinomas. Ann Surg Oncol 2014;21(2):621–8.
17. Sun W, Song L, Ai T, Zhang Y, Gao Y, Cui J. Prognostic value of MET, cyclin D1 and MET gene copy number in non-small cell lung cancer. J Biomed Res 2013;27(3):220–30.
18. Jurmeister P, Lenze D, Berg E, et al. Parallel screening for ALK, MET and ROS1 alterations in non-small cell lung cancer with implications for daily routine testing. Lung Cancer 2015;87(2):122–9.
19. Schildhaus HU, Schultheis AM, Rüschoff J, et al. Met amplification status in therapy-naïve adeno- And squamous cell carcinomas of the lung. Clin Cancer Res 2015;21(4):907–15.

20. Li A, Niu F-Y, Han J-F, et al. Predictive and prognostic value of de novo MET expression in patients with advanced non-small-cell lung cancer. Lung Cancer 2015;90(3):375–80.

21. Park S, Koh J, Kim DW, et al. MET amplification, protein expression, and mutations in pulmonary adenocarcinoma. Lung Cancer 2015;90(3):381–7.

22. Weingertner N, Meyer N, Voegeli A-C, et al. Correlation between MET protein expression and MET gene copy number in a Caucasian cohort of non-small cell lung cancers according to the new IASLC/ATS/ERS classification. Pathology 2015;47(June):1.

23. Tran TN, Selinger CI, Kohonen-Corish MRJ, et al. Alterations of MET Gene Copy Number and Protein Expression in Primary Non-Small-Cell Lung Cancer and Corresponding Nodal Metastases. Clin Lung Cancer 2016;17(1):30–8.

24. Watermann I, Schmitt B, Stellmacher F, et al. Improved diagnostics targeting c-MET in non-small cell lung cancer: expression, amplification and activation? Diagn Pathol 2015;10:130.

25. Wang X, Song N, Zhang Y, et al. Coexpression of c-Met and Notch-1 correlates with poor prognosis in resected non-small-cell lung cancer. Tumor Biol 2015;36(9):7053–9.

26. Gao HF, Li AN, Yang JJ, et al. Soluble c-Met Levels Correlated With Tissue c-Met Protein Expression in Patients With Advanced Non???Small-Cell Lung Cancer. Clin Lung Cancer 2017;18(1):85–91.

27. Noonan SA, Berry L, Lu X, et al. Identifying the appropriate FISH criteria for defining MET copy number-driven lung adenocarcinoma through oncogene overlap analysis. J Thorac Oncol 2016;11(8):1293–304.

28. Tong JH, Yeung SF, Chan AWH, et al. MET Amplification and Exon 14 Splice Site Mutation Define Unique Molecular Subgroups of Non-Small Cell Lung Carcinoma with Poor Prognosis. Clin Cancer Res 2016;22(12):3048–56.

29. Awad MM, Oxnard GR, Jackman DM, et al. MET exon 14 mutations in Non-small-cell lung cancer are associated with advanced age and stage-dependent MET genomic amplification and c-Met overexpression. J Clin Oncol 2016;34(7):721–30.

30. Lapère C, Cortot AB, Grégoire V, Cockenpot V, Tulasne D, Copin M-C. Preferential Localization of MET Expression at the Invasion Front and in Spreading Cells Through Air Spaces in Non–Small Cell Lung Carcinomas. Am J Surg Pathol 2017;41(3):414–22.

31. Koeppen H, Yu W, Zha J, et al. Biomarker analyses from a placebo-controlled phase II study evaluating erlotinib??onartuzumab in advanced non-small cell lung cancer: MET expression levels are predictive of patient benefit. Clin Cancer Res 2014;20(17):4488–98.

32. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. N Engl J Med 2012;366(26):2443–2454.

33. Gettinger SN, Horn L, Gandhi L, et al. Overall Survival and Long-Term Safety of Nivolumab (Anti-Programmed Death 1 Antibody, BMS-936558, ONO-4538) in Patients With Previously Treated Advanced Non-Small-Cell Lung Cancer. J Clin Oncol 2015;33(18):2004–12.

34. Gettinger S, Rizvi NA, Chow LQ, et al. Nivolumab Monotherapy for First-Line Treatment of Advanced Non-Small-Cell Lung Cancer. J Clin Oncol 2016;34[1] Gett(25):2980–7.

35. Rizvi NA, Mazières J, Planchard D, et al. Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): A phase 2, single-arm trial. Lancet Oncol 2015;16(3):257–65.

36. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non–Small-Cell Lung Cancer. N Engl J Med 2015;373(2):123–35.

37. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non–Small-Cell Lung Cancer. N Engl J Med 2015;373(17):1627–39.

38. Carbone DP, Reck M, Paz-Ares L, et al. First-Line Nivolumab in Stage IV or Recurrent Non–Small-Cell Lung Cancer. N Engl J Med 2017;376(25):2415–26.

39. Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the Treatment of Non–Small-Cell Lung Cancer. N Engl J Med 2015;372(21):2018–28.

40. Hui R, Garon EB, Goldman JW, et al. Pembrolizumab as first-line therapy for patients with PD-L1-positive advanced non-small cell lung cancer: a phase 1 trial. Ann Oncol Off J Eur Soc Med Oncol 2017;28(4):874–81.

41. Herbst RS, Baas P, Kim D-WW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): A randomised controlled trial. Lancet 2016;387(10027):1540–50.

42. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Pembrolizumab versus Chemotherapy for PD-L1–Positive Non–Small-Cell Lung Cancer. N Engl J Med 2016;375(19):1823–33.

43. Herbst RS, Soria J-C, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014;515(7528):563–7.

44. Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. Lancet 2016;387(10030):1837–1846.

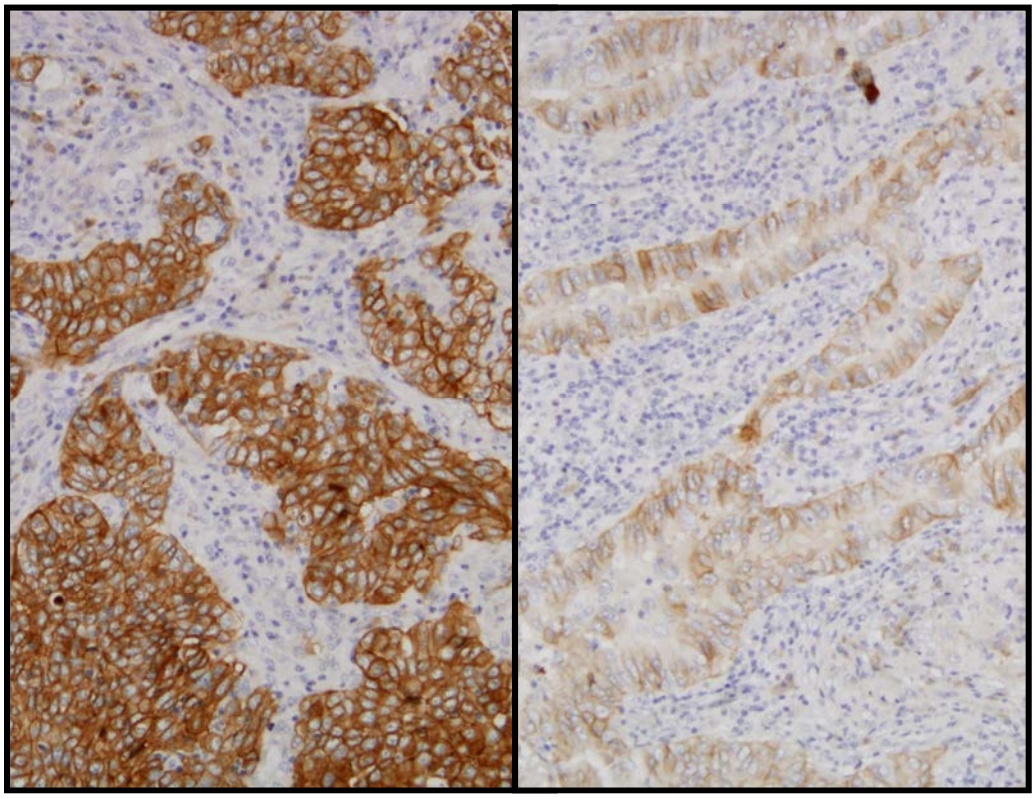
45. Rittmeyer A, Barlesi F, Waterkamp D, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet 2017;389(10066):255–65.

46. Peters S, Gettinger S, Johnson ML, et al. Phase II Trial of Atezolizumab As First-Line or Subsequent Therapy for Patients With Programmed Death-Ligand 1-Selected Advanced Non-Small-Cell Lung Cancer (BIRCH). J Clin Oncol 2017;35(24):2781–9.

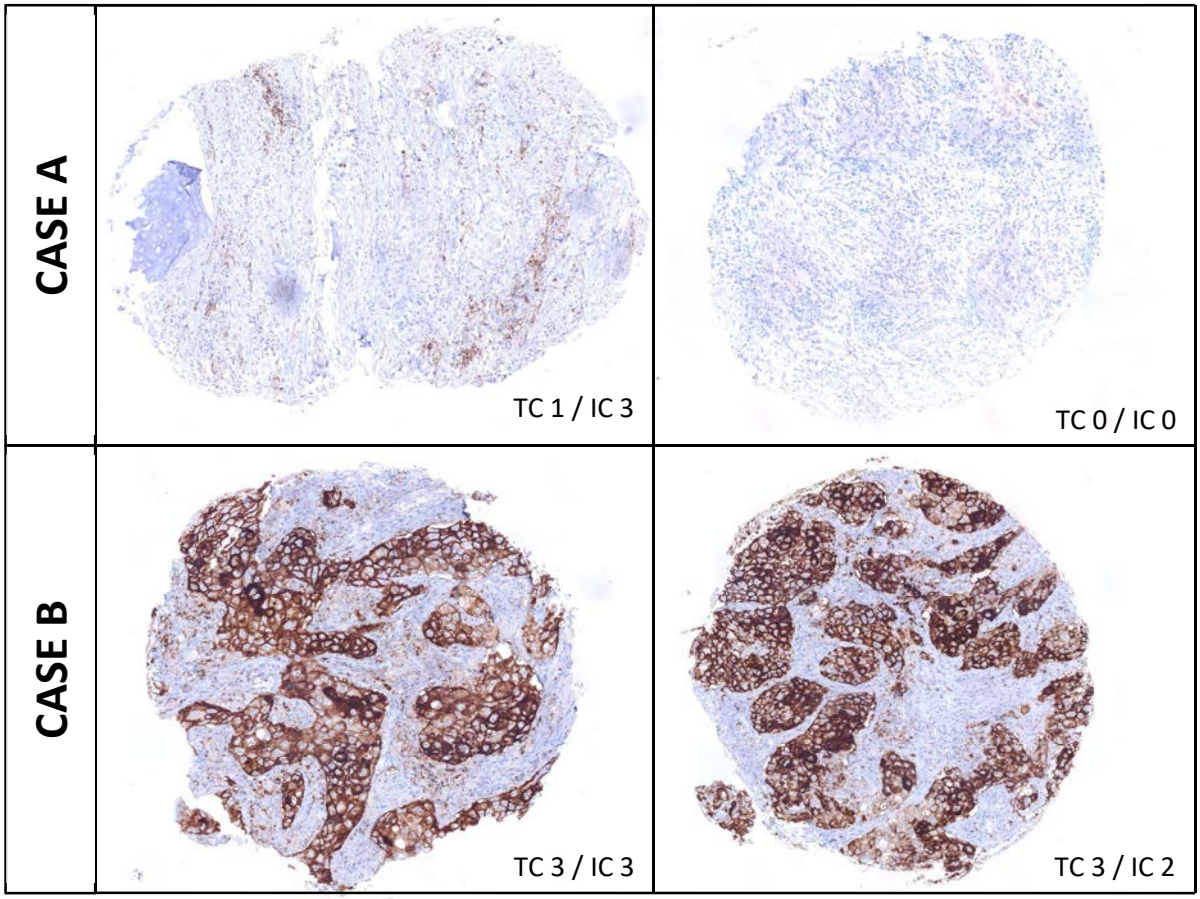
47. Garassino M, Vansteenkiste J, Kim J-H, et al. PL04a.03: Durvalumab in ≥3rd-Line Locally Advanced or Metastatic, EGFR/ALK Wild-Type NSCLC: Results from the Phase 2 ATLANTIC Study. J Thorac Oncol 2017;12(1):S10–1.

48. Gulley JL, Rajan A, Spigel DR, et al. Avelumab for patients with previously treated metastatic or recurrent non-small-cell lung cancer (JAVELIN Solid Tumor): Dose-expansion cohort of a multicentre, open-label, phase 1b trial. Lancet Oncol 2017;2045(17):1–12.

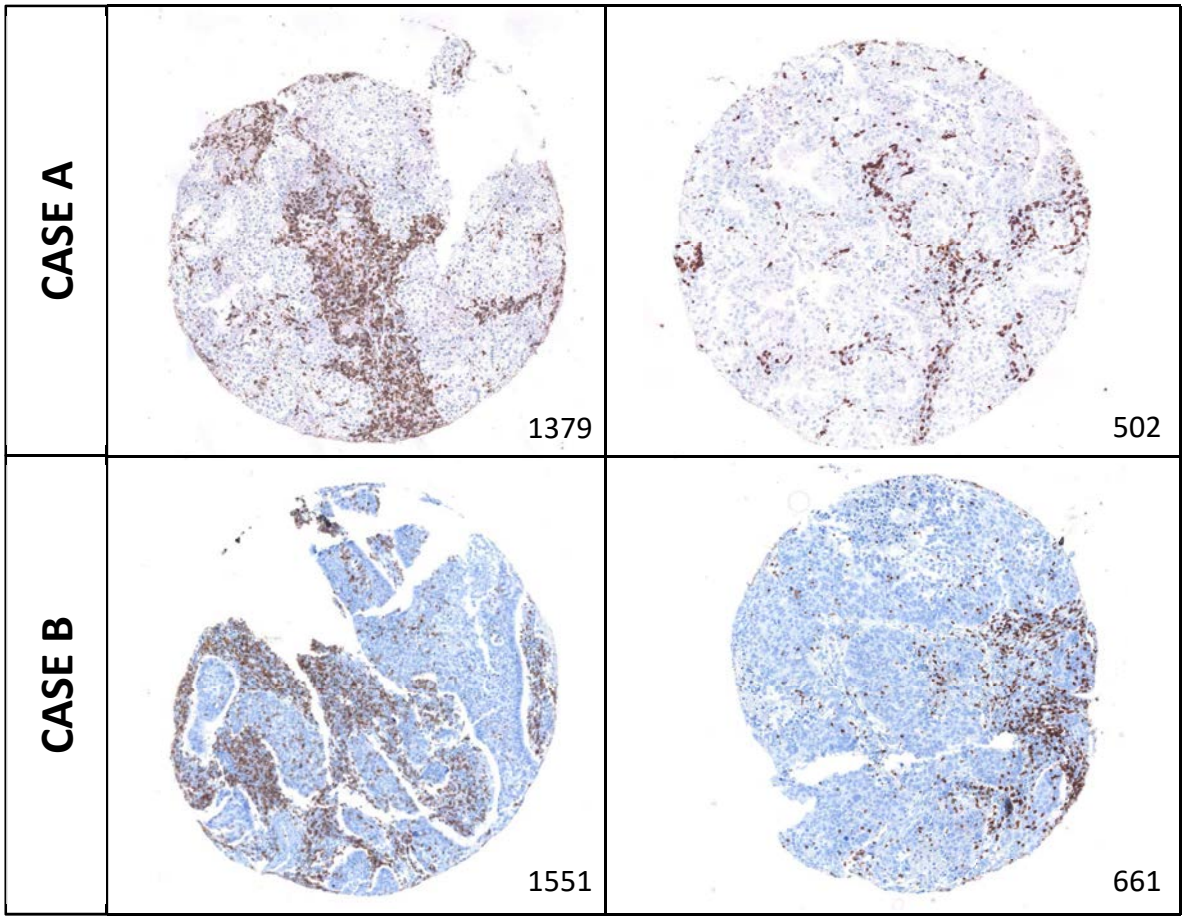
9. APPENDIX II. Complementary Images



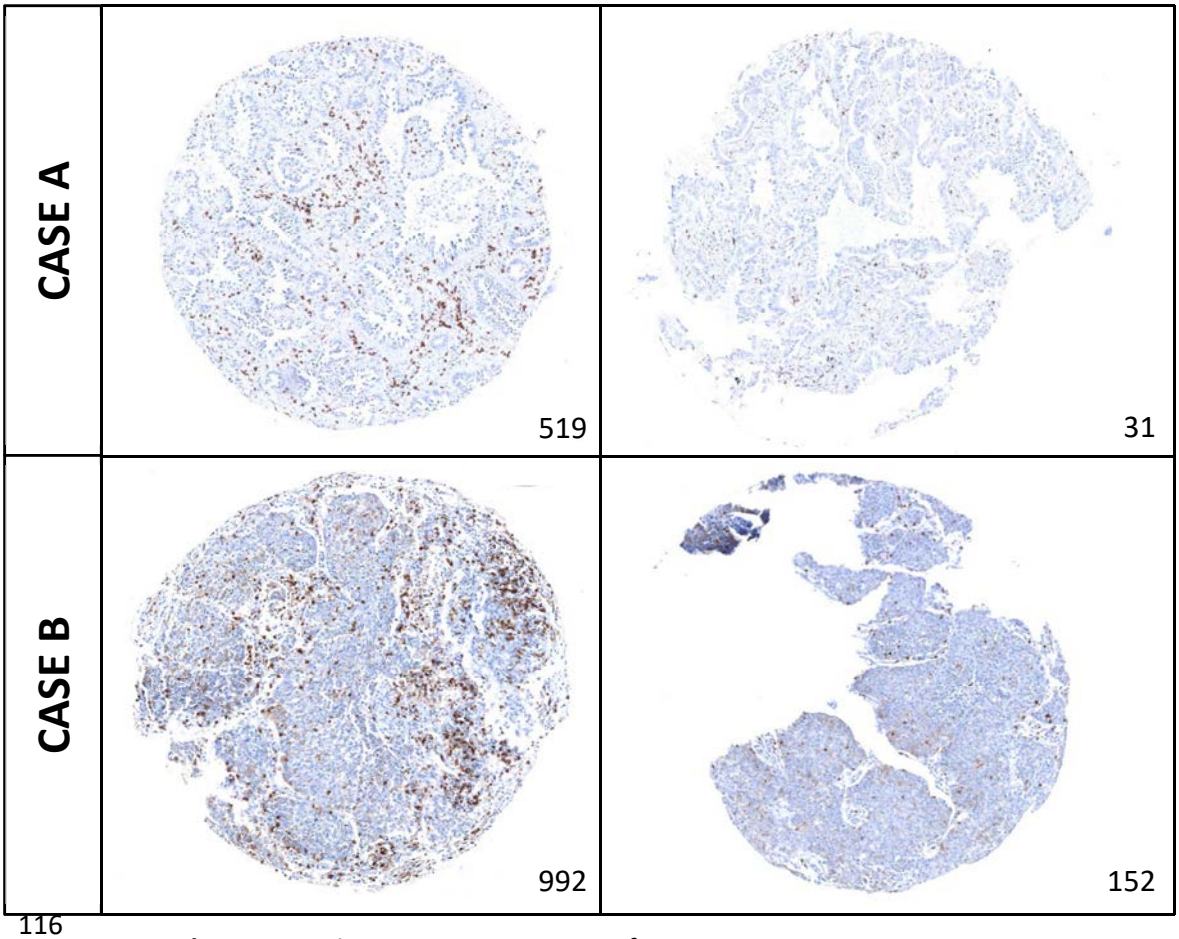
Complementary Figure 1. Heterogeneity of MET expression. Right photo: Solid pattern +4 in 100% of tumor cells. Left photo: Acinar pattern +2 in 40% of cells.



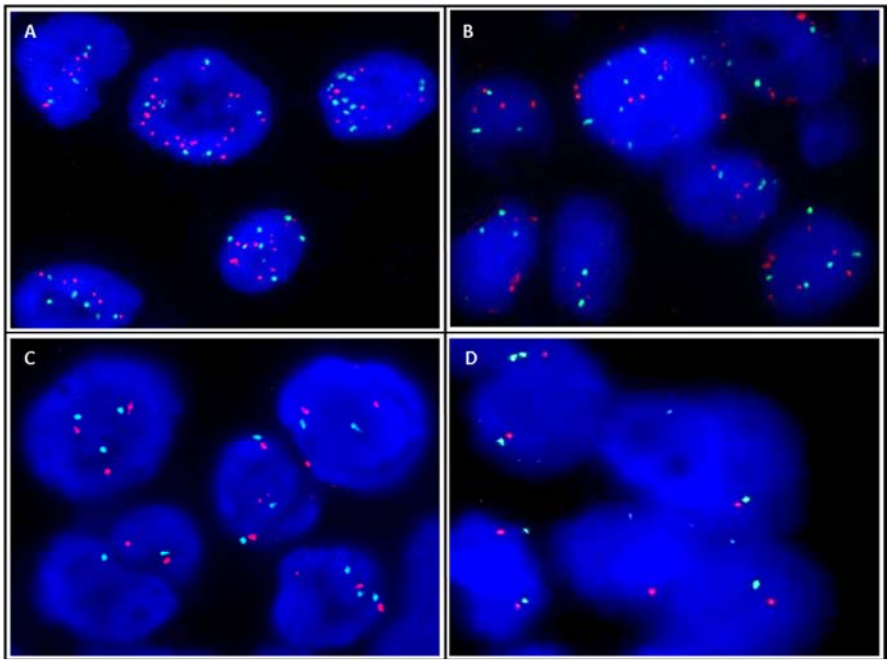
Complementary Figure 2. Heterogeneity of PD-L1 expression in immune cells.



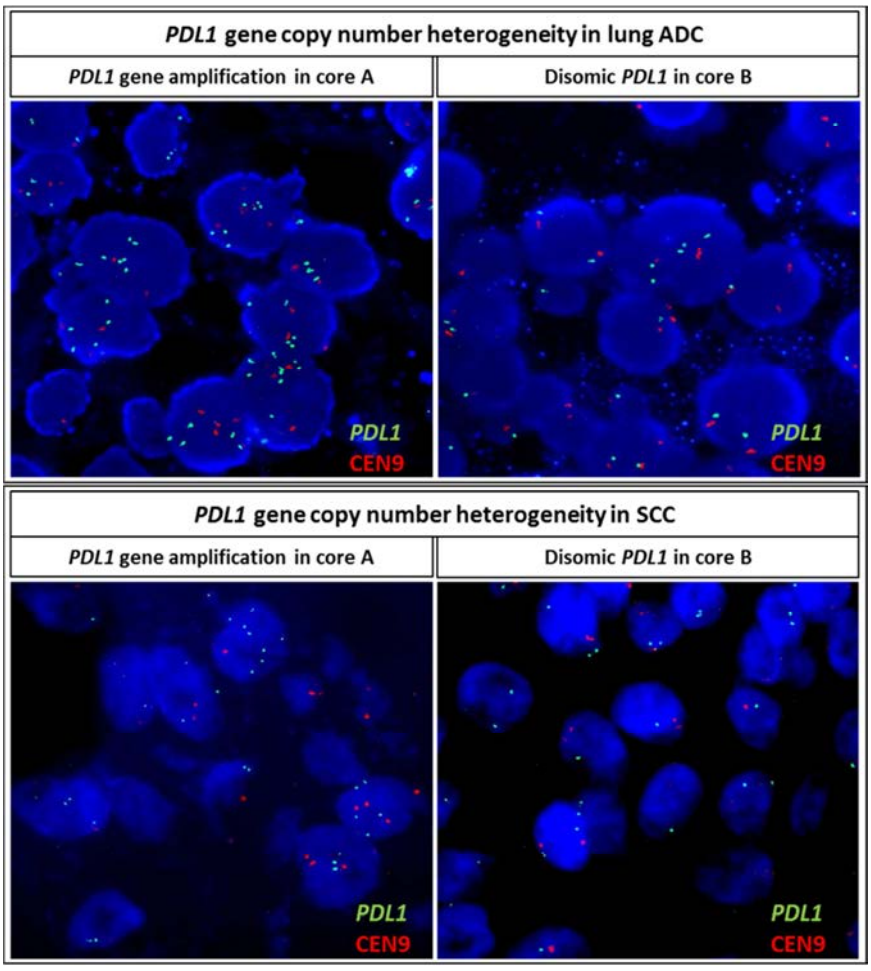
Complementary Figure 3. Heterogeneity of CD3 counts.



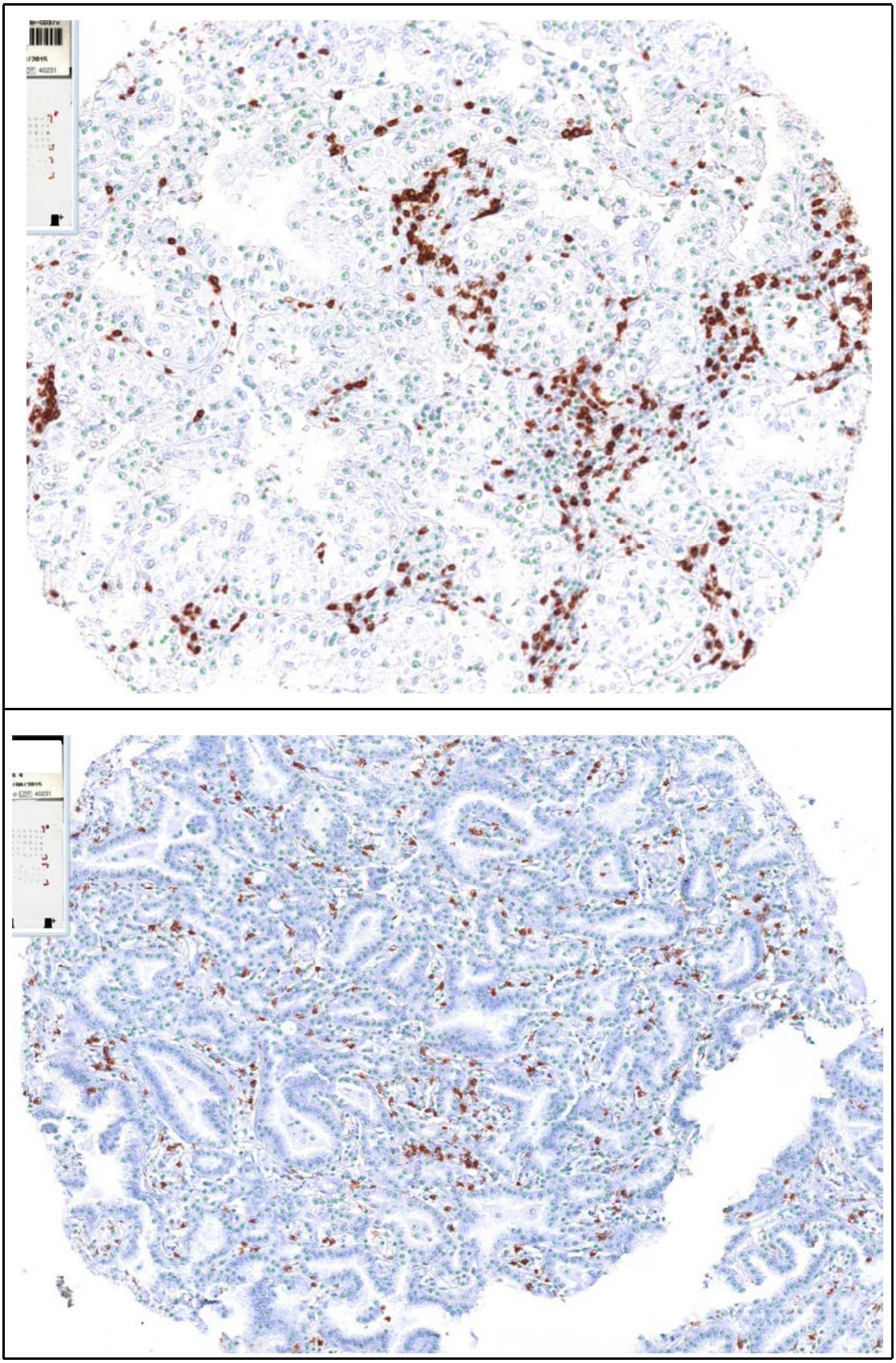
Complementary Figure 4. Heterogeneity of CD8 counts.



Complementary Figure 5. Reproduction of Supplementary Figure 1 of the first study. Four tumor samples exhibiting different *MET*/CEP7 FISH signal patterns: **A.** *MET* positive nuclei showing a *MET*/CEP7 ratio ≥ 2 (truly amplified). **B.** *MET* positive nuclei showing a *MET* ≥ 5 (high polysomy). **C.** *MET* negative nuclei showing ≥ 2.5 *MET* copies (classified as *MET* gain). **D.** *MET* negative disomic pattern.



Complementary Figure 6. Different *PDL1* gene patterns.



Complementary Figure 7. Examples of CD3 (Top) and CD8 (Bottom) quantitation. Stained cells are highlighted with a red spot and normal nuclei with a green spot. Top picture: 502 stained over 1695 unstained cells. Bottom picture: 304 stained over 4049 unstained cells.