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MOLECULAR TARGET THERAPY AND IMMUNOTHERAPY OF RARE LUNG TUMORS

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Alla mia famiglia e a Federico che con il loro amore mi hanno sempre supportato in tutte le mie avventure spronandomi a dare il meglio di me stessa.

A tutte le persone che mi hanno aiutato a crescere.

ABSTRACT

Introduction

Lung cancer is an heterogeneous disease, with 1-2% of rare histological subtypes. Moreover, even the more common histologies such as adenocarcinoma and squamous cell carcinomas are furtherly divided according to their molecularalterations, with up to 727 mutated genes identified in the COSMIC database, and taking into account these sub-classifications is crucial for the development of more effective and personalized therapies.

New molecular profiling technologies, such as next generation sequencing (NGS), have revolutionized the assessment of molecular alteration in clinical practice. The diverse molecular and clinical landscape of Non-Small Cell Lung Cancer (NSCLC) is transforming the once frequent disease in a archipelago of rare diseases, characterized by their own specific treatment and outcomes and needing tailored approach even in the clinical trial setting.

Moreover, immune-checkpoint blockers have emerged as a new approach to cancer treatment, only marginally dependent from the molecular characterization. However, their efficacy in rare histological subtypes is currently unknown.

Methods

We analyzed a prospective cohort of 1408 advanced NSCLC patients treated at the Sant'Orsola- Malpighi University Hospital from 2019 to 2021 and submitted to NGS for molecular characterization at the time of diagnosis. This NGS analysis was performed using the Oncomine focus Thermo Fischer panel.

3 of them were included in CRETA trial evaluating cabozantinib in RET rearranged NSCLC

Finally, in the same period we collected all cases of non-adenocarcinoma and non SCC NSCLC finding 14 cases who included in CHANCE trial, evaluating atezolizumab in rare histologies.

Results

We analyzed 1408 patients. Of them, 410 (29%) had rare alteration (RET 3%, NTRK 0,2%, FGFR1 2%, MET exon14 skipping 3%, BRAF V600 4%, ALK fusion EGFR exon 20 2%) and 36 (2%) had a uncommon mutation. 20 patients with rare alteration received a treatment within a clinical trial.

7 RET- rearranged patients J2G-MC-JZJC clinical trials assessing selective RET-inhibitors. Also, another 7 patients tested positive for the BRAF V6006 mutation and have been enrolled in the Array clinical trial assessing a combination of anti-BRAF and anti-MEK agents. Patients with KRAS (Gly12Cys), FGFR1-4 mutation, MET skipping ex14 mutations, were eligible for other ongoing open studies such as Amgen 20190009 comparing efficacy of sotorasib vs docetaxel, Fight-207 assessing activity of pemigatinib and CINC280J12201assessing activity of the novel met inhibitor capmatinib .

Identification of patients with rare molecular alterations helped in the selection of personalized therapy that are currently undergoing clinical trials.

We therefore have activated the CRETA clinical trial, a multicenter study evaluating the efficacy and safety of cabozantinib in patients with RET- rearranged where and 5 patients have been enrolled in Italy.

There have currently been few clinical cases on the use of ICI agents in individuals with rare lung cancer due to their low incidence. In 2018 we joined the CHANCE clinical trial, a multicenter study evaluating the efficacy and safety of atezolizumab in patients with rare lung cancer histologies where and 14 patients have been so far enrolled in the Bologna site.

Conclusion

Our study shows that performing NGS in addition to histological subtyping on A-NSCLC at the time of diagnosis is feasible and allows to identify several distinct molecularly-defined cancer subgroups which are candidate for specific tailored treatments within clinical practice and/or clinical trials which may significantly improve clinical outcome.

ABBREVIATIONS

PAHs	polycyclic aromatic hydrocarbons	Rb	retinoblastoma
GWA	genome-wide association	FVC	forced vital capacity
HNBs	heat-not-burn	SV40	monkey vacuolating virus
HPV	human papilloma virus	BKV	polyomavirus BK
JCV	polyomavirus JC	HCMV	human cytomegalovirus
NSCLC	non-small cell lung cancer	SCLC	Small cell carcinoma
AC	Adenocarcinoma	TTF-1	Thyroid Transcription Factor-1
CK7	cytokeratin 7	EBV	Epstein-Barr virus
AIS	Adenocarcinoma in situ	MIA	Minimally invasive adenocarcinoma
AI	Invasive adenocarcinoma	SqCC	Squamous cell carcinoma
LCC	Large cell carcinoma	TAFs	tumor-associated fibroblasts
MMP1	Matrix Metallopeptidase 1	CT	computed tomography
FLAC	Fetal adenocarcinoma	CCF	Spindle cell carcinoma

GCC	Giant cell carcinoma	TEM	epithelium-mesenchyme transition
ACC	Cystic adenoid carcinomas	PACC	Primary pulmonary adenoid cystic carcinoma
p-EMC	Primary lung epithelial myoepithelial carcinoma	SMA	smooth muscle actin
EMC	epithelial myoepithelial carcinoma	LELC	Primary pulmonary lymphoepithelioma-like carcinoma
NUT	nuclear protein in testis	PET	positron emission tomography
TNM	tumor-node-metastasis	BAL	bronchial washings
AJCC	American Joint Committee on Cancer	cTNM	clinical staging
pTNM	pathological staging	ycTNM	clinical staging after neo-adjuvant therapy
PI3K	phosphatidylinositol 3-kinase	mTOR	mammalian target of rapamycin
MAPK	mitogen activated protein kinase	TKIs	tyrosine kinase inhibitors
RECIST	Response Evaluation Criteria In Solid Tumors	PFS	progression-free survival
WHO	World Health Organization	KRAS	Kirsten's rat sarcoma
ypTNM	pathological staging after neo-adjuvant therapy	EGFR	epidermal growth factor receptor

IHC	Immunohistochemistry	ALK	anaplastic lymphoma kinase
BRAF	murine sarcoma V-raf viral oncogene B	TTF-1	thyroid transcription factor-1
NCCN	National Complete Cancer Network	ESMO	European Society of Medical Oncology
EMA	European Medicines Agency	JNK	c-Jun N-terminal kinase
NGS	next generation sequencing	CTC	circulating tumor cells
CTLA-4	cytotoxic T-cell-associated antigen 4	APCs	antigen presenting cells
ICIs	immune checkpoint inhibitors	OS	overall survivals
RET	REarranged during Transfection	ERK	extracellular signal-regulated kinase
ctDNA	circulating tumor DNA	PD-1	programmed cell death 1
DOC	docetaxel	PD-L1	Programmed death-ligand 1

INTRODUCTION

Epidemiology

In recent decades, much has been understood about the mechanisms that regulate tumor progression, including knowledge of how the immune system supports this growth, and lung cancer remains a leading cause of cancer worldwide[1]. According to the 2020 GLOBOCAN report, 2.21 million new cases of lung cancer (11.4% of total cases) and 1.79 million deaths (18.0% of cancer deaths) were estimated[2].

The correlation between smoking habits and lung cancer incidence is well known. Despite growing public awareness of tobacco-associated risk and recent improvements in early diagnosis, approximately one-third of patients present with clinically evident metastatic disease.[3]

Furthermore, in many countries with a low-medium socio-economic situation and a high rate of active smokers, they coincide with the highest number of cancer diagnoses. [4]

Italy is no exception, with 41,000 new diagnoses of lung cancer in 2020 which made it the third cancer by incidence both in males (15%, after prostate and colorectal cancer), and in females (6 %, after breast and colorectal cancer).

Compared to previous years, women appear to have a higher incidence (+ 2.5% compared to 2019) of lung cancers, as smoking has increased significantly.

Survival is strongly influenced by prevention and secondary therapy. Through an early diagnosis there is a greater chance of being treated effectively, with a benefit in terms of reducing specific mortality. Currently, 5-year survival data are similar to the European trend reported by EUROCARE-5 (15% male 19% female)[5]

Risk factors

Lung cancer risk factors can be divided into two groups: genetic and environmental factors.

The former are factors dependent on the "genetic profile of the host".

An example is rs1048943 (CYP1A1) a phase 1 enzyme that plays an important role in the metabolism of many endogenous substrates such as steroids and eicosanoids and exogenous carcinogens such as polycyclic aromatic hydrocarbons (PAHs), aromatic and heterocyclic amines which can then covalently bind to DNA forming DNA adducts, thus initiating the carcinogenic process in many extrahepatic tissues, including the lung. [6]

Identification of genetic polymorphisms underlying lung cancer risk has been the subject of recent intra-genome association studies (GWAs). The 15q25 susceptibility region contains three nicotine cholinergic receptor genes (CHRNA3, CHRNA5, and CHRNB4), which encode nicotinic acetylcholine receptors both at the neuronal level and in other tissues. These variants are associated with increased vulnerability to tobacco addiction and altered smoking behavior, including an increase in the number of cigarettes smoked per day[7]. The risk of lung cancer is also increased in the alteration of Rb (retinoblastoma) and the framework of the Li-Fraumeni syndrome, characterized by germline mutations in the tumor suppressor gene p53 [8].

Environmental factors can be infectious, chemical, physical, diet, lifestyle, and habits, etc..

One of the main risk factors for lung cancer is cigarette smoking. International Cancer Research has identified about 4,000 chemical constituents in tobacco smoke. Also interesting is the presence among these carcinogens of radioactive materials such as radon and its decay products, bismuth, and polonium. The mechanisms of carcinogenesis act over time include the formation of DNA adducts, their metabolites, and the damage of free radicals, therefore cancer increases with age, the duration of smoking, and the number of cigarettes smoked per day. The relative risk of lung cancer in long-term smokers was estimated to be 10 to 30 times that of non-smokers. This risk decreases in former smokers and a favorable effect of discontinuation is also evident for later cessation in life.

Epidemiological evidence and biological plausibility support a causal association between exposure to passive smoking and lung cancer risk in nonsmokers, ranging from 20% to 30%.[9]

In recent years, other types of cigarettes have spread, including electronic nicotine delivery systems (ENDS). ENDS allows the liquid to be heated through an atomizer, to create an aerosol containing nicotine and substances such as flavors, propylene glycol, vegetable glycerin, and other ingredients that the user inhales. Among these substances, propylene glycol has been shown to be irritating to the upper airways, decreasing both the forced vital capacity (FVC) and the forced expiratory volume in 1 second (FEV1)[10]. During the use of the electronic cigarette, numerous substances are produced including formaldehyde, a group 1 carcinogen according to the IARC classification (International

Agency for Research on Cancer), group 2B acetaldehyde and, in low concentrations, nitrosamines NNN and NNK of tobacco. In addition to heavy metals, nickel, cadmium, and lead are also present irritants such as acrolein and carbonyl compounds [11].

Recently the tobacco industry is promoting a new product, the heat-not-burn (HNBs) tobacco products branded as IQOS. A comparative analysis showed that HNBs also massively reduce levels of carbon monoxide (CO), tar, and carcinogenic compounds, while maintaining the nicotine concentrations found in conventional cigarette smoke (combustion).

However, HNBs need to undergo ongoing cleaning maintenance a recent study found that charring increases if devices are not cleaned after heating tobacco sticks. Additionally, melting of the polymer film filter (at 90˚C) has been reported to release formaldehyde cyanohydrin, a toxic substance of concern. [12]

Although such a heart-not-burn cigarette would always be less harmful than conventional cigarettes, it must be remembered that it encourages nicotine addiction and makes smokers desist from quitting, moreover it does not reduce cardiovascular risks.

Occupational or environmental exposures to carcinogenic elements such as heavy metals and atmospheric pollutants are correlated with the onset of this neoplasm and in particular aluminum, beryllium, chromium, cadmium, polycyclic aromatic hydrocarbons, vinyl chloride and arsenic are substances classified as group 1 by the IARC as a human carcinogen.

Even radon, produced by the radioactive decay of radium 226 can produce neoplastic transformation due to the emission of alpha particles. If inhaled, alpha radiation is highly damaging to tissues, including the respiratory epithelium as it induces cell damage.

Another important environmental factor is air pollution, in particular exposure to PM (PM2.5 Cu, PM10 S, PM10 Ni, PM10 Zn, PM10 K), which is associated with an increased risk of lung cancer and mortality regardless of cigarette smoking.

Finally, viral factors can also induce a greater risk of developing lung cancer, for example, viruses such as human papillomavirus (HPV), monkey vacuolating virus (SV40), polyomavirus BK (BKV), polyomavirus JC (JCV) and human cytomegalovirus (HCMV) all have oncogenic capacity. They can alter cell cycle processes, increasing replication and inhibiting apoptosis. [13]

Other risk factors of lesser importance are states of chronic lung inflammation, such as chronic bronchitis and pulmonary emphysema, which are also caused by smoking, and tuberculosis for which it has been shown associated with the development of carcinoma and the presence of scarring. [14]

Histopathology

Lung cancer is classified according to 2015 WHO classification of lung cancers.[15]

Lung carcinomas are divided into two large groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).

Small cell lung carcinoma (SCLC) makes up about 15% -20% of lung cancers. It most frequently affects the male sex, the 6th and 7th decade of life and is related to cigarette smoking; in fact, only in 1% of cases does it occurs in non-smokers. Histologically it is characterized by small monomorphic cells with scarce cytoplasm and with a rounded or fusiform nucleus and a small and scarcely visible nucleolus.). From the histological point of view there are different histological types, a high degree of malignancy due to the

precocity giving loco-regional and distant metastases, which almost always makes it radically incurable already at the time of diagnosis.

Approximately 80% of patients have the histological subtypes known as non-small cell lung cancer (NSCLC), which is composed by lung adenocarcinoma (AC), squamous cell lung cancer (SCC) and Large cell carcinoma (LCC) are the most common subtypes.

Lung adenocarcinoma

Adenocarcinoma (AC) is an invasive malignant epithelial tumor that accounts for 30-45% of lung cancers. Tumors tend to arise peripherally compared to the large airways, at the level of the smaller caliber bronchi often with pleural involvement and consensual neoplastic effusion, moreover, they can often arise near areas of parenchymal scarring.

L'AC represents the most frequent form in non-smokers in females and men under the age of 50. Diagnosis is based on the histological observation of glands composed of columnar epithelial cells and/or the presence of mucinous cells; in some cases, "bezel ring cells" are observed, characterized by the presence of abundant mucus in the cytoplasm with a nucleus located in the periphery. ACs often show histological heterogeneity for this reason it has different subgroups: acinar (more differentiated), papillary (compact appearance), ex-bronchioloalveolar (pleomorphic), and solid with mucus production. The histochemical diagnosis involves the positivity for molecules such as TTF- 1 (Thyroid Transcription Factor-1), CK7 (cytokeratin 7), and napsin A.

Local spread may involve direct spread to the pleura, diaphragm, pericardium, or bronchi with advanced disease that spreads to the mediastinum, great vessels, trachea, esophagus, spine, or adjacent lobe. However, AC often causes distant metastasis,

particularly extension to a contralateral lobe, pleural nodules, malignant pleural or pericardial effusion or any distant site such as the brain, bones, or liver.

From the immunohistochemical point of view, it is classified into:

Adenocarcinoma in situ (AIS) is characterized by size of less than 3 cm, and has no stromal, vascular, and pleural invasion. In the majority of cases, this involves nuclear atypia and the lack of invasive adenocarcinoma patterns and the absence of aerial dissemination. On CT, these lesions appear as ground-glass opacities, sometimes difficult to distinguish from inflammatory changes in the lungs.

- Minimally invasive adenocarcinoma (MIA) usually presents as a solitary nodule with a size of 3 cm, with predominantly lepid growth, stromal infiltration of less than 0.5 cm, absence of necrosis and vascular and pleural invasion. It too is divided into mucinous and non-mucinous. The invasive stromal foci may have acinar, papillary, micropapillary or solid architecture or be made up of single cells dispersed in the desmoplastic stroma.
- Invasive adenocarcinoma (AI); is characterized by a size greater than 1-3 cm, with a stromal invasion greater than 0.5 cm, It includes different subtypes, classified according to grade: low-grade lepidic adenocarcinoma (G1), papillary and acinar carcinoma intermediate grade (G2) and high grade (G3), solid and micropapillary carcinoma.

Rare phenotypes will be discussed later. Of these, AIS and MIA have better outcomes when resected early [16].

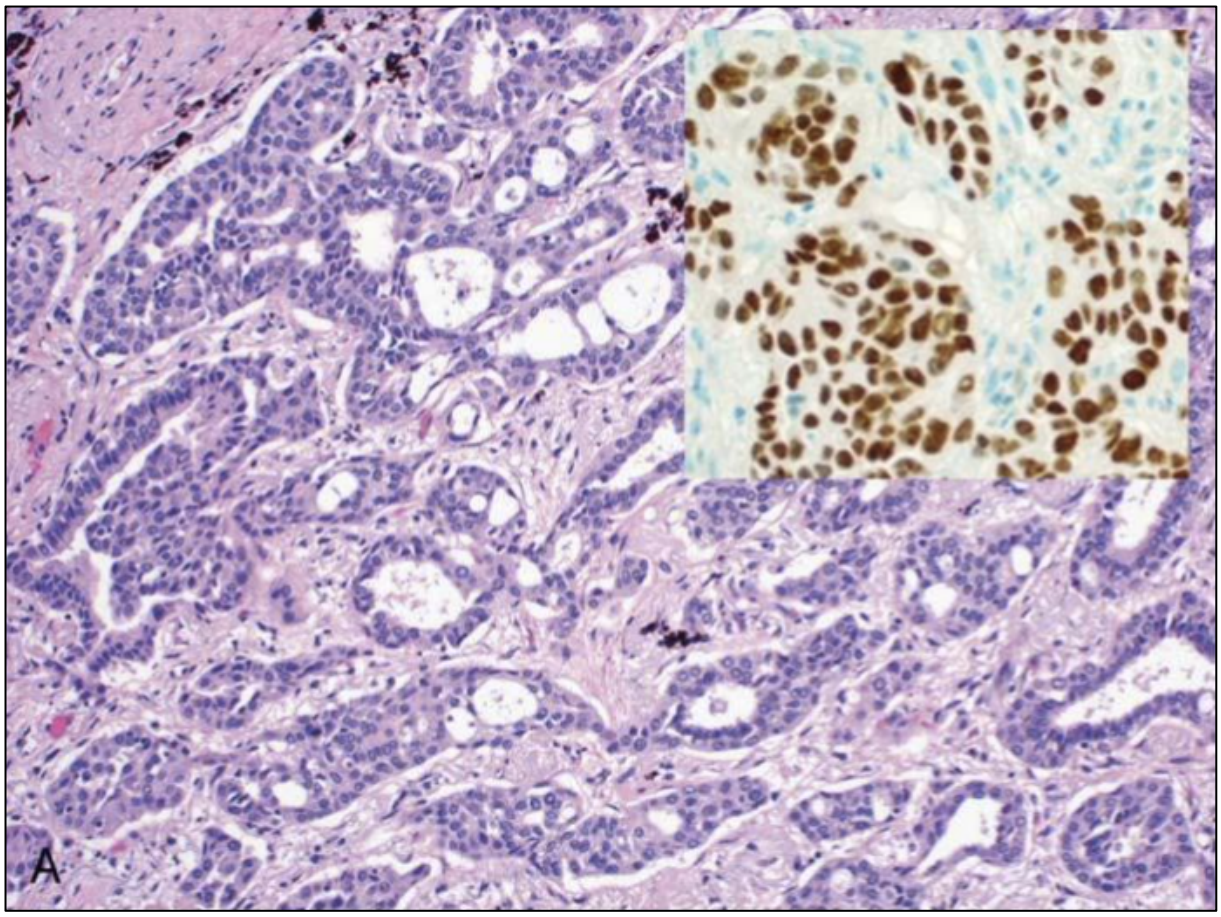

Figure 1- Lung adenocarcinoma: the upper right box shows the expression of the thyroid transcription factor (TTF-1), identified by immunohistochemistry[17].

Lung squamous cell carcinoma (SqCC)

Squamous cell carcinoma (SqCC) is a malignant epithelial tumor strongly associated with smoking with an incidence of 30% -45%. On the cell membrane there are cellular spines or desmosomes that connect the adiA cells, for this histotype three variants have been identified: the keratinizing type, non-keratinizing type and the basaloid form[18].

The immunohistochemical profile is characterized by the positivity for TP63 (p63), p40, cytokeratins (CK5 / 6) and desmocollin-3[19]. Bronchoscopy and biopsy represent the gold standard of diagnosis. SCCs typically arise centrally in the lung, hilar region, and proximal bronchi. They tend to have a locally aggressive growth pattern. On the contrary, it is much

slower in passing from in situ to invasive cancer, taking up to 4 years. Consequently, distant metastases occur less frequently than in AC.

This neoplasm appears as a cauliflower mass, white greyish, with a hard and lobulated consistency with the presence of necrosis and hemorrhage that can cavitate the tumor mass.

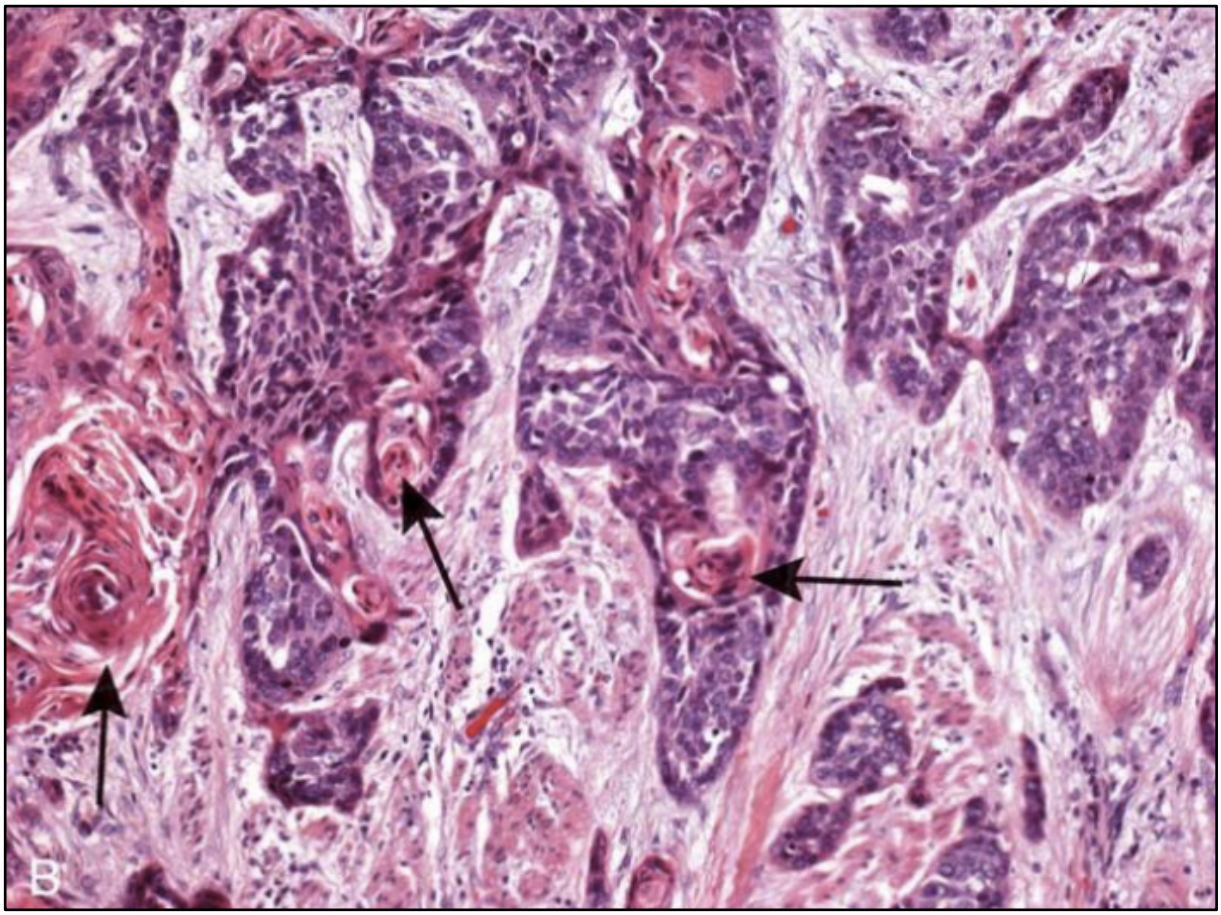

Figure 2 - Lung squamous cell carcinoma (SqCC) with keratinization (arrows)[17].

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Large cell carcinoma

Large cell carcinoma (LCC) is a heterogeneous histological subtype of NSCLC.

It is a disease characterized by large, and atypical cells with a moderate amount of cytoplasm in the absence of specific signs of differentiation. Biopsies and histological samples do not allow a correct diagnosis as NSCLC often has specific morphological criteria therefore this diagnosis is only allowed on surgical samples.

Based on genetic and immunohistochemical studies, most cases of NSCLC previously classified as LCC are now reclassified, according to the WHO 2015 classification[20]. Consequently, depending on the outcome of TTF-1 or P40 staining, the LCCs were reclassified as adenocarcinoma or non-keratinized squamous cell carcinoma[21]. As a result, this reclassification reduced the diagnosis of LCC which currently accounts for 1-2% of all lung cancer cases.

Natasha Rekhtman et al. stratified 102 large cell carcinomas by immunohistochemistry for TTF-1 and DNp63 / p40 as classifiers of LCC adenocarcinoma (LCC-ADC) and LCC squamous cell carcinoma (LCC-SCC), respectively, if such classifiers were absent the subtype is indicated with LLC-null [22].

In this study, they showed that LCC-null disease-free survival and OS were significantly lower than LCC-ADC and LCC-SCC making the LLC-null phenotype more aggressive. [23].

Recently in some studies it has been shown that tumor-associated fibroblasts (TAFs) extracted from LCC tumors go into premature senescence. M. Gabasa et al demonstrated how LCC overexpress Matrix Metallopeptidase 1 (MMP1) which, in addition to having a role in promoting tumor invasion and extracellular matrix remodeling processes, has a key role in the paracrine senescence of TAFs and promotes the most common phenotype. aggressive of the LCC [24,25].

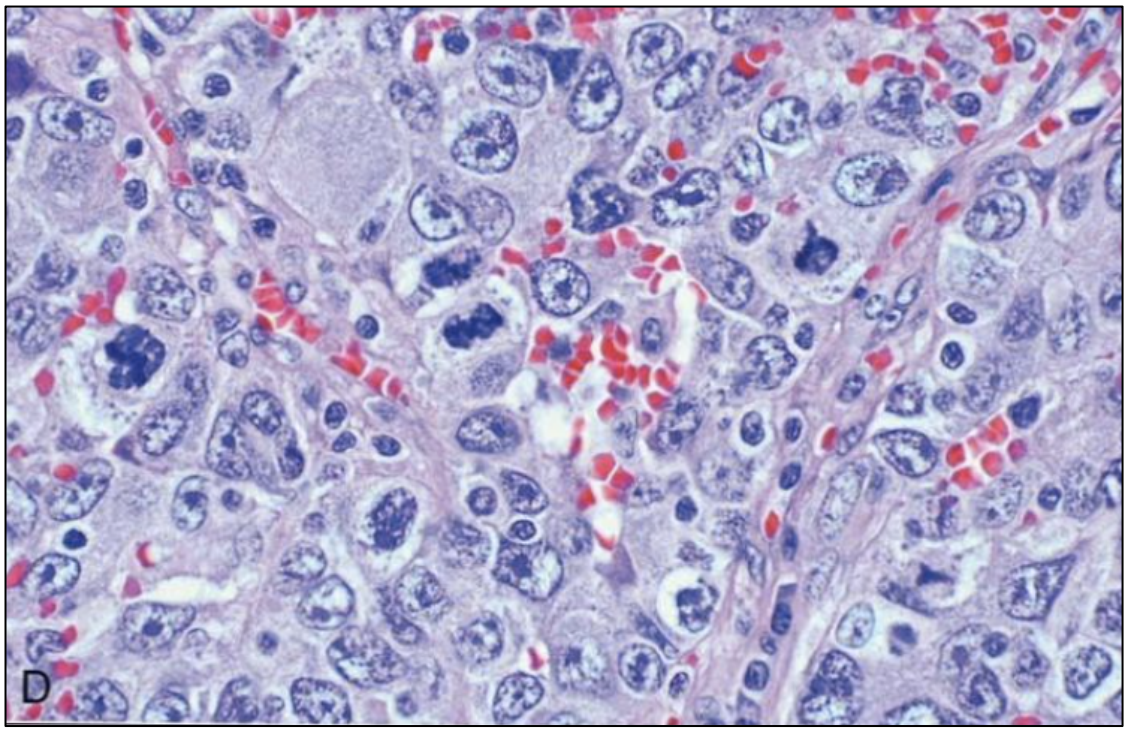

Figure 3-Large cell carcinoma are pleomorphic and show no signs of squamous or glandular differentiation[17]
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Rare Histological subtypes

Colloid carcinoma

Primary colloidal adenocarcinoma is classified as an extremely rare variant of invasive adenocarcinoma (0.24% of all lung cancers) and was previously defined as mucinous cystadenocarcinoma of the lung. The term "colloidal carcinoma" was first used in 1992 and is characterized by abundant extracellular mucin and scant neoplastic epithelium[20]. The extracellular mucin pool takes the place in the normal lung parenchyma. The fibro-connective stroma lined with epithelium is present in small quantities and "floats" within the mucin areas. Only some tumors have a small percentage of solid parts characterized by conventional adenocarcinoma. The dimensions are irregular and vary from a few cm to more than 10 cm in the largest diameter[26]. Diagnosis is usually based on magnetic resonance imaging (MRI) and computed tomography (CT) imaging[27]. Biological behavior is generally unknown and appears to have a favorable clinical course compared to other primary lung cancers.

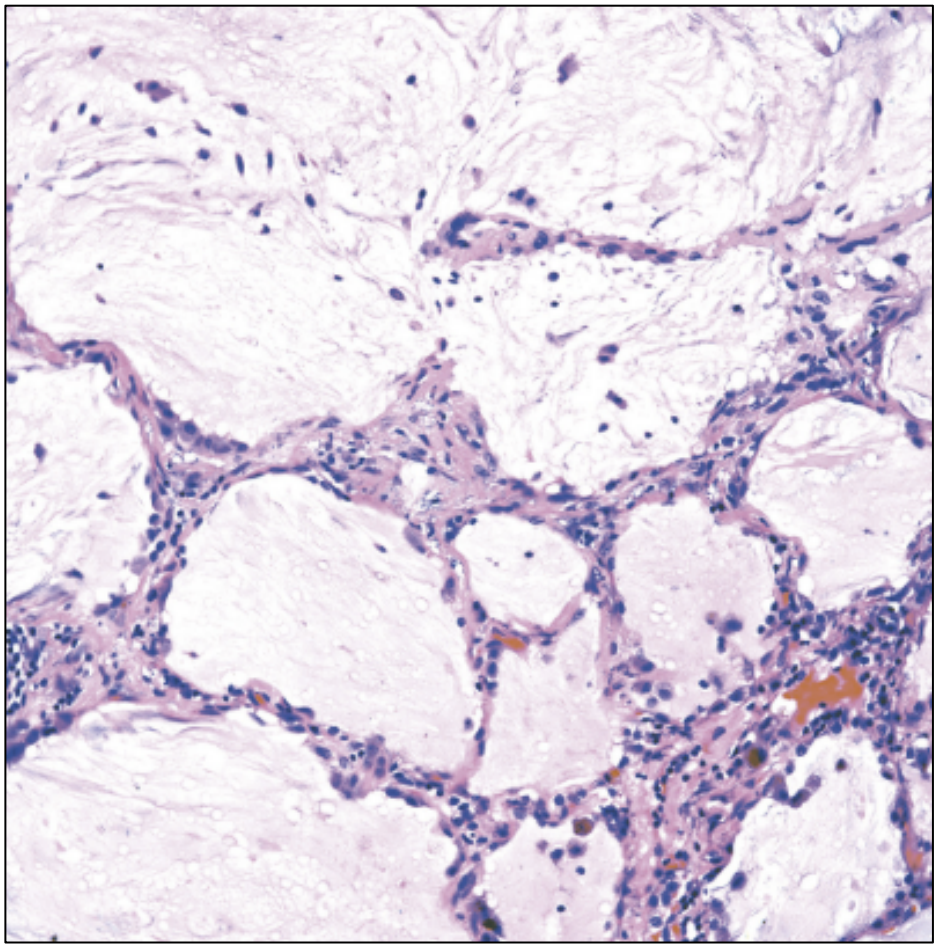


Figure 4-Colloid carcinoma of lung is a subtype that shows abundant extracellular mucin[26].

Fetal adenocarcinoma

Fetal adenocarcinoma (FLAC) is a rare (0.5%) subtype of primary lung adenocarcinoma first described by WG Branard in 1945, while we have to wait until 1999 for the current classification by the World Organization of Healthcare (WHO)[28]. It is a biphasic tumor, it morphologically resembles the early secretory endometry, in fact it has several tubular glandular branches lined with non-ciliary glycogen-rich colonnar cells immersed in a loose cellular fibroblastic stroma.

Given their morphological diversity, they are divided into low-grade (L-FLAC) and high-grade (H-FLAC) tumors, based on different clinico-pathological characteristics and biological behaviors.

The former arise solitary in young patients (from the third to the fourth decade of life) in stage I-II with lower mortality risks than the counterpart. H-FLACs occur predominantly in advanced stage III-IV elderly men (sixth and seventh decade of life) with a history of significant smoking and over-expression of p53 [29] However, compared to conventional lung adenocarcinoma, a low frequency of EGFR and KRAS mutations in FLAC tumors were found in several studies.

In the CT scan and in the radiography the lesion appears with a heterogeneous aspect in fact there are soft tissues and areas of necrosis and has a diameter between 2 and 12 cm. About 30-40% of patients have an asymptomatic disease.

Surgical resection is the standard treatment for FLACs. Although having limited effect, radiotherapy and chemotherapy have been used as adjuvant therapy.

According to Nakatani et al, L-FLACs have a better prognosis than conventional lung adenocarcinoma of the same grade, while H-FLACs have a worse prognosis [30].

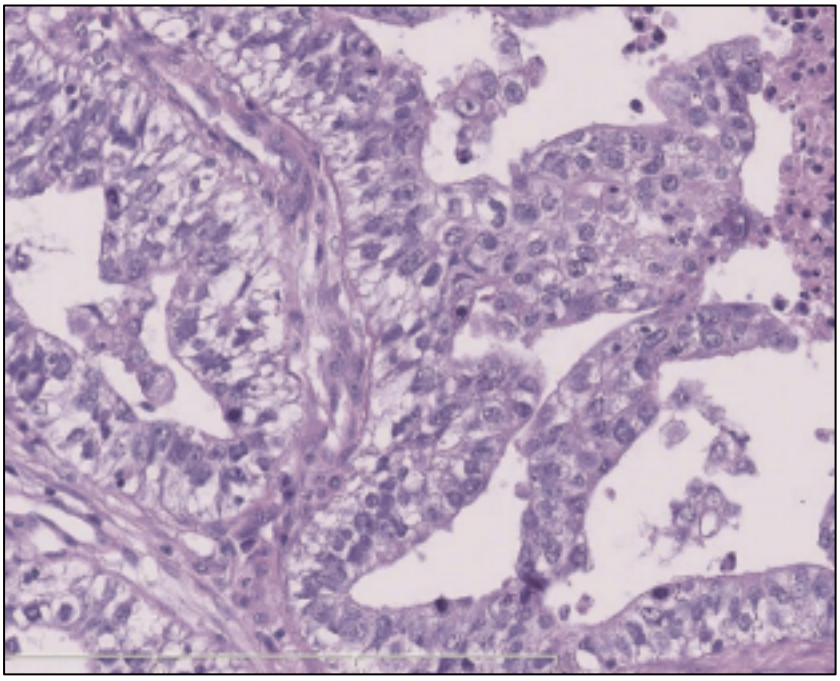


Figure 5-Fetal adenocarcinoma[29]

Sarcomatoid carcinoma

Primary lung sarcomatoid carcinomas (SC) include a rare and heterogeneous group of NSCLC and bronchial carcinomas accounting for no more than 3% of all lung cancer cases. They are not lung-specific and can be seen in other organs such as the head and neck and digestive system. It is cancer that mainly affects elderly men, the main risk factor being tobacco or occupational exposures such as asbestos and chemicals.

The current WHO classification of lung cancers defines sarcomatoid carcinoma as poorly differentiated NSCLC containing a sarcoma component or sarcoma-like differentiation.

Histologically it is composed of giant cells of variable size, multinucleated with pleomorphic and hyperchromatic nuclei, eosinophilic cytoplasm, and high nucleus-cytoplasm ratio.

It is a very heterogeneous tumor, in fact, it could take on a sarcomatoid appearance with organized spindle cells or carcinoma-like [31].

According to the 2015 classification, the term "sarcomatoid carcinoma" includes 5 subtypes that can be diagnosed by examining surgical samples.

- Spindle cell carcinoma (CCF) is composed of an almost pure population of fusiform epithelial cells.

- Giant cell carcinoma (GCC) is composed almost entirely of giant tumor cells.

The other three tumor subtypes have a dual nature also called biphasic;

-Pleomorphic carcinoma is the predominant subtype it covers 70-80% of sarcomatoid carcinomas and is an association between a pseudosarcomatous portion and one (about 10%) carcinomatous (cells spindle and/or giants).

-Carcinosarcoma is defined by the association of a heterologous sarcomatous portion and a carcinomatous portion.

-Pulmonary blastoma is extremely rare <0.1%, and consists of embryonic-type epithelial elements (typically low-grade fetal adenocarcinoma) and primary mesenchymal stroma [20].

Sarcomatoid carcinoma is associated with a high rate of progression under first-line platinum-based chemotherapy and has a very poor prognosis.

Several studies show that these tumors have overexpression of c-MET which plays a key role in the biological process of epithelium-mesenchyme transition (TEM) observed in sarcomatoid carcinoma. TEM is involved in tumor invasion and metastatic spread. The activation of this oncogenic pathway suggests the use of targeted therapy such as crizotinib in patients refractory to chemotherapy [32]. The treatment of SC patients with ICI by evaluating the abundance of PDL-1 PD1 has also been shown to be effective in terms of high response rates and prolonged overall survival[33].

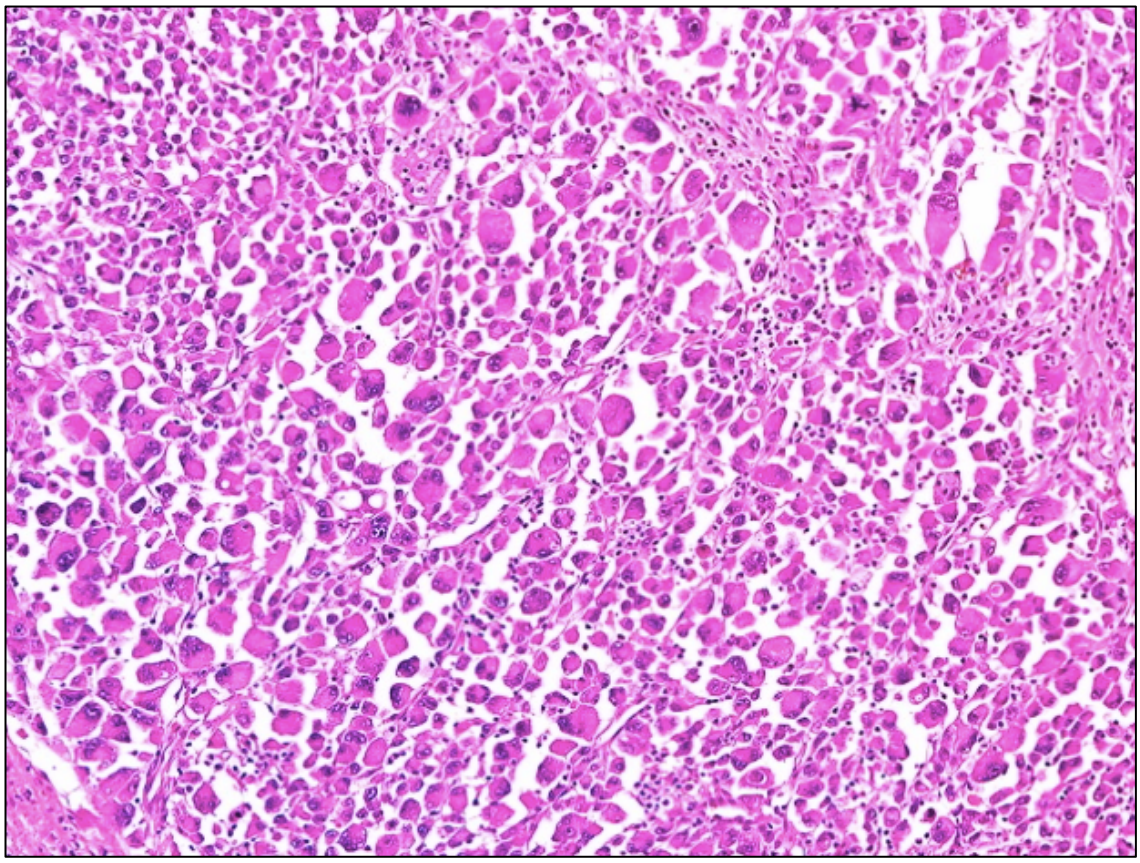


Figure 6 - Giant Cell Carcinoma of Lung[34]

Primary mucoepidermoid carcinoma

Primary mucoepidermoid carcinoma of the lung is a rare malignancy comprising <1% of all lung malignancies. It is a form of the squamous type. Which arises mostly in the salivary glands and in the central bronchial region. Bronchopulmonary cancer consists of the presence of mucipary cells, epidermoid cells and intermediate cells. Based on histological characteristics, the presence of atypia and necrosis, number of mitoses, bronchopulmonary mucoepidermoid carcinomas are classified into low grade and high grade. The former have a well-differentiated shape, have a cystic appearance with a delimited diameter <4 cm[35].

Several studies show the different biological behavior of these two tumors; high-grade mucoepithelioid carcinomas are then aggressive, while low-grade tumors have an excellent prognosis after surgical treatment.

Mucoepidermoid carcinoma is often associated with the presence of the fusion protein MECT1-MAML2 which is considered a genetic driver event in its pathogenesis and suggestive of diagnosis [36].

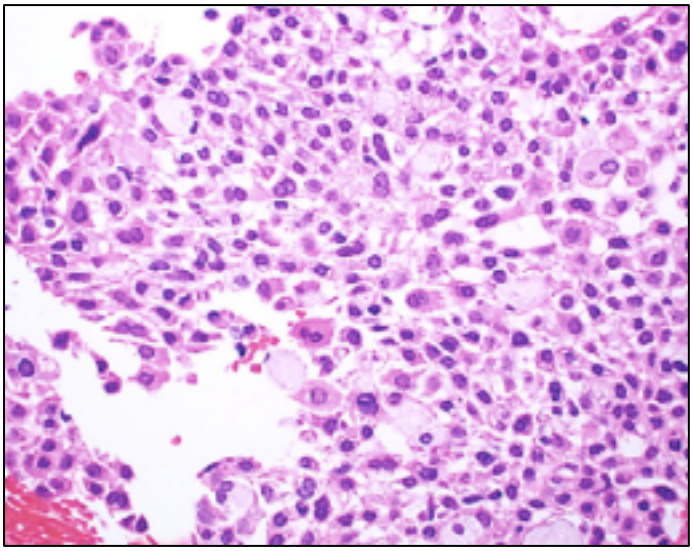


Figure 7- Histopathologic image of primary mucoepidermoid carcinoma of the major salivary gland. H & E stain.[35]

Primary pulmonary adenoid cystic carcinoma

Cystic adenoid carcinomas (ACC) are classified as a subtype of NSCLC. They frequently originate from the salivary glands of the head and neck, but can also affect the skin, breast, upper digestive system and lungs.

The main histological subtypes are fibrosis, tumor, and solid latter is believed to be associated with a more aggressive phenotype[37].

ACCs grow mainly from mixed seromucosal glands in the tracheal lumen and have a slower growth rate and rarely develop distant lymph nodes and metastases (M). The treatment of choice for patients with ACC is complete surgical resection, although incomplete resection (R1, R2) is often used due to the location of the tumor and its invasiveness. Patients with positive margins undergo adjuvant radiotherapy with a multimodal therapeutic approach with an OS at 5 and 10 years of 94% and 91%.[38]

Primary pulmonary adenoid cystic carcinoma (PACC) is a particularly rare disease and accounts for only 0.04-0.2% of all primary lung cancers. PACC occur in younger, but nonsmoking patients. The first therapeutic strategy is complete surgical resection, as far as possible. This tumor has a notable tendency to local recurrence and late hematogenous metastases. The 3, 5, and 10-year overall survival rates are 92.9, 91.7, and 70.0%, respectively[35].

Advanced PACC's palliative chemotherapy has rarely been described in the literature, however, Ming-Ming Hu et. Al have shown that some patients with PACC may marginally benefit from dual platinum-based chemotherapy.

Some EGFR mutated patients have been treated with EGFR-TKI but such mutations are very rare in salivary gland carcinomas [39].

Activation of MYB has recently been described in 80% of ACC cases, including PACC, in particular the chromosomal translocation t (6; 9) (q22–23; p23–24) with consequent MYB-NFIB fusion gene is frequent [40].

Other studies identify possible biomarkers in ACC and PACC tumors, however major genes implicated in NSCLC such as EGFR, KRAS, BRAF, ALK, PIK3CA, PDGFRA and DDR2

may not be lead genes in PACC.34. Considering these data, it must be researched a new therapeutic strategy for advanced PACC.

A recent study has shown that checkpoint inhibitor drugs can have a limited impact in the treatment of localized ACC of the lacrimal gland, but pretreatment with radiation can improve the tumor's response to these drugs [41]. Still nothing is known about the treatment of PACC with checkpoint inhibitor drugs.

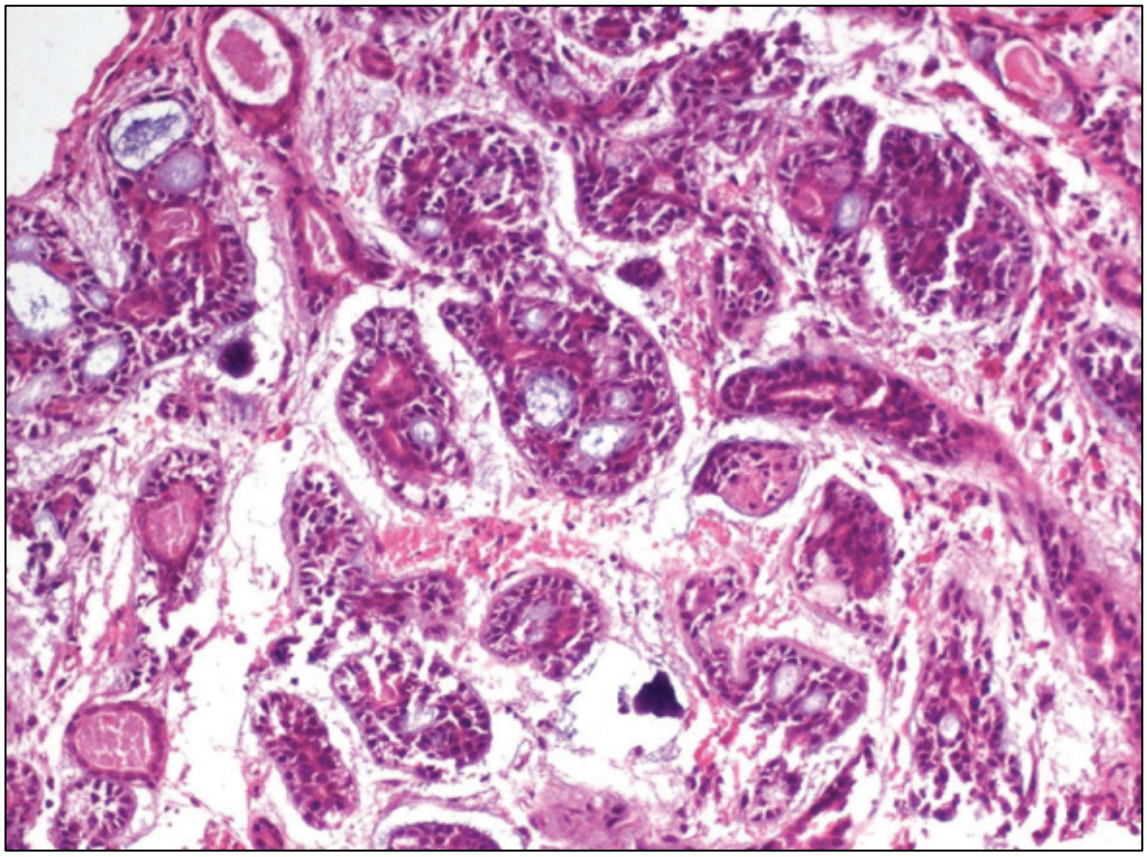


Figure 8- Histopathological image of Primary pulmonary adenoid cystic carcinoma. H & E stain[42].

Primary pulmonary epithelial-myoepithelial carcinoma

Primary lung epithelial myoepithelial carcinoma (P-EMC) is a rare subgroup of salivary gland-type lung cancers. The site of onset of EMC is the parotid gland, followed by the major and minor salivary glands, lacrimal gland, and seromucinous glands in the sinus tract or bronchi.

P-EMC is a tumor characterized by biphasic morphology, the epithelial cells form the duct and multilayer polygonal cells positive for epithelial markers, while the external cells are positive for myoepithelial markers such as protein S100 and smooth muscle actin (SMA), p63 and cytokeratin 5/6[43,44].

Unusual cytological and architectural features have also occasionally been described such as a cribrose growth pattern, basaloid appearance, papillary-cystic pattern, and sebaceous differentiation [45].

The diagnosis occurs through preoperative bronchoscopy, typically it is found in a uniform population of the sex towards the sixth and seventh decade of life. However, epithelial myoepithelial carcinoma (EMC) may show hybrid features of cystic adenoid carcinomas (AdCC). Justin A. Bishop et al. They demonstrated that 40-65% of AdCCs are characterized by MYB-NFIB fusion, while EMC are devoid of it, demonstrating that the classical histological approach supported by MYB FISH can be a valid differential [46]. In the past decade, EMC mucoepidermoid carcinoma has been found to harbor characteristic translocations such as (CRTC1-MAML2 or CRTC3-MAML2)[47–49]. Bundel et al. recently used the same approach as Justin A. Bishop using the presence of MAML2 fusions as a differential diagnosis tool to highlight the EMC with acinar differentiation [50].

EMC occasionally from distant metastases and relapses, they can be successfully managed by bronchoscopic tumor ablation [46]. The estimated five-year and ten-year Disease-survival rates were 90%, 95%, and 80-90%, respectively. [51]

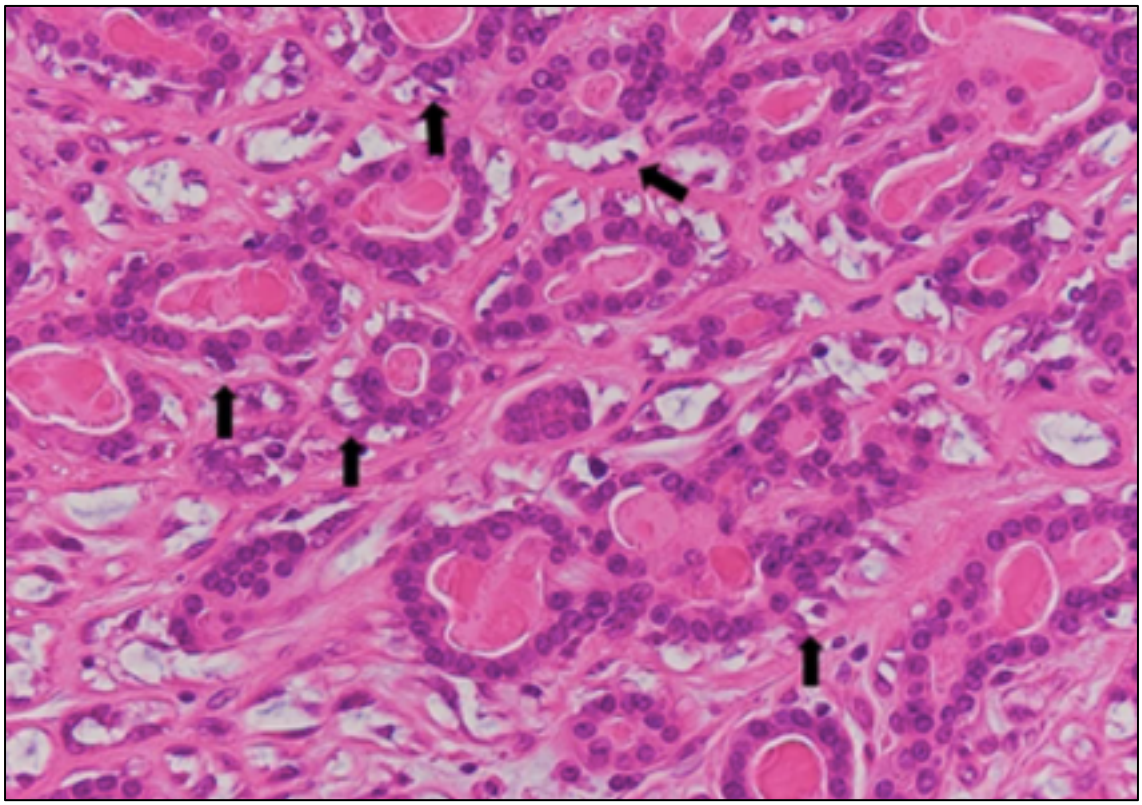


Figure 9-Primary pulmonary epithelial-myoepithelial carcinoma H&E stain. The duct-like structures comprised two distinct cell layers[52].

Lymphoepithelioma-like carcinoma

Primary pulmonary lymphoepithelioma-like carcinoma (LELC) is another rare lung cancer subtype, classified as a form of large cell carcinoma according to the World Health Organization classification [53].

It was first described in 1987 by Begin et al. such as an Epstein-Barr virus (EBV) - associated epithelial neoplasia [54].

It occurs more often in younger patients with no history of smoking and is associated with a better prognosis than other lung cancer histologies. It is usually diagnosed early and resectable (stage I or II). In these states, the recommended therapeutic option is complete resection, while multimodal therapy, chemotherapy and palliative radiotherapy is used for locally advanced and metastatic stages [55] .

Histologically, the lung LELC is made up of undifferentiated carcinoma cells. The fibrous stroma is rich in reactive lymphoplasmacytic cells and other inflammatory cells that infiltrate the tumor islands recalled by a wide expression of the transcripts of the chemotactic protein 1 of monocytes [56,57].

Zhanhong Xie et al. have shown how LELCs present a high expression of PD-L1 and the lack of canonical mutations of the main molecular drivers. Furthermore, the expression of PD-L1 correlated with the abundance of lymphocytes in the stroma [58].

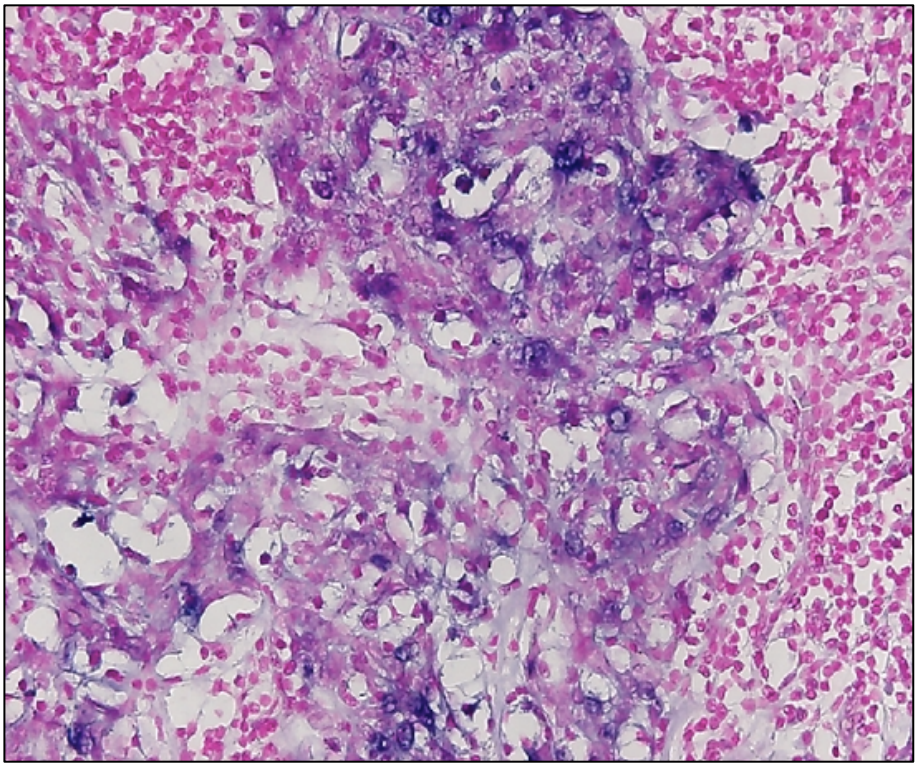


Figure 10- Lymphoepithelioma-like carcinoma, abbreviated LELC. H&E stain[59].

NUT carcinoma

NUT (nuclear protein in testis) carcinomas of the lung or mediastinum are poorly differentiated, and highly aggressive tumors genetically defined by the presence of NUT gene rearrangement and the most common form of fusion is BRD4-NUT. Aerodigestive tract and mediastinum are the most affected anatomical sites [60,61].

NUT carcinoma occurs mainly in children and young adults and can affect people of any age. Surgery and radiation therapy are the main treatment options for NUT carcinomas. Poor prognosis is the main feature of NUT carcinoma, Giridhar et al. found a median overall survival (OS) of 5 months from diagnosis with OS of 24.99% at 1 year and 7.09% at 5 years [62,63].

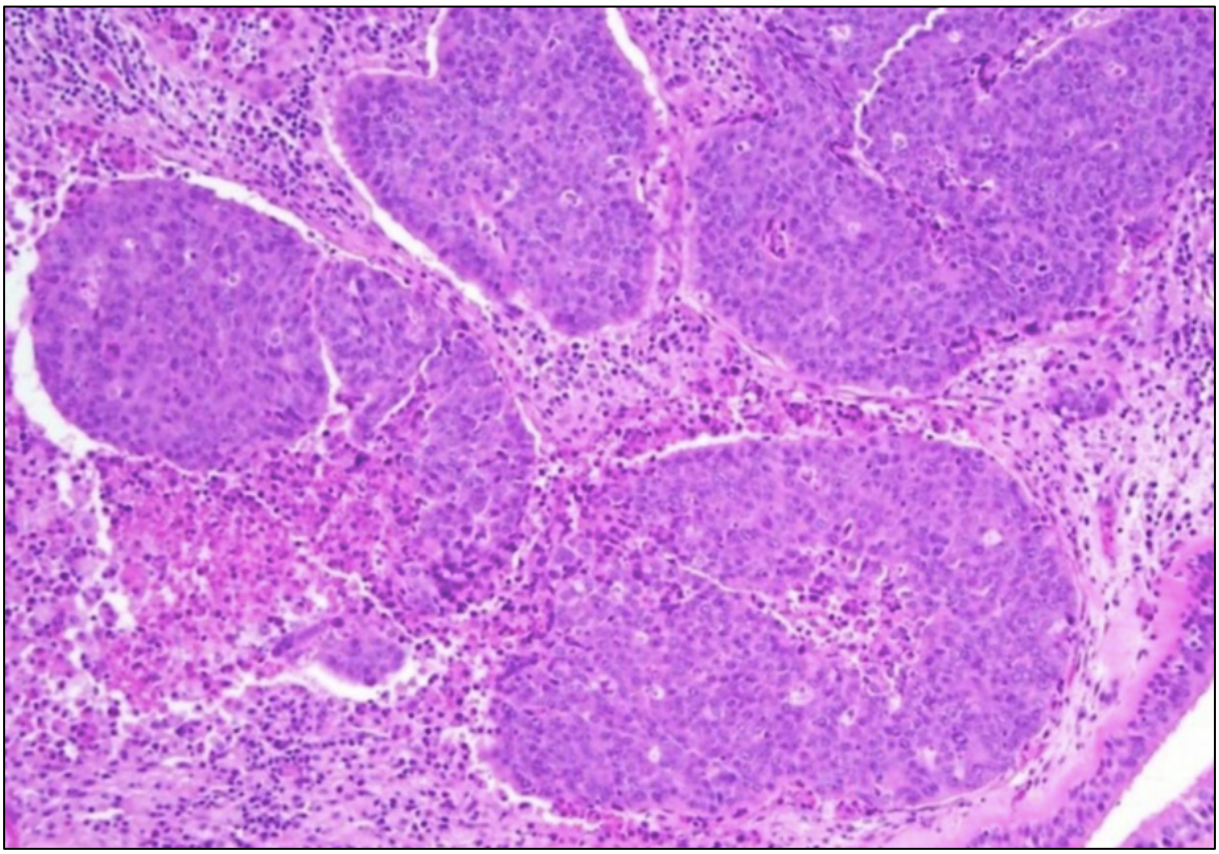


Figure 11-NUT carcinoma[64]

Diagnosis

Patients with clinical suspicion of lung cancer present with both general symptoms such as fatigue and weight loss and symptoms that may be related to the primary tumour such as persistent cancer, dyspnea and hemoptysis[65]. Patients should be immediately referred for radiological imaging for clinical staging. If abnormalities are found, mediate computed tomography (CT) and positron emission tomography (PET) with and without contrast medium are evaluated. At the same time, a biopsy is acquired to obtain a cyto-histological evaluation to confirm the diagnosis of lung cancer and to do the molecular analysis. These examinations are then reinforced MRI of the brain, chest or spine / pelvis and mediastinoscopy are modalities used to achieve a final clinical staging. For patients with lung cancer and who have signs and symptoms of skeletal involvement, technetium 99 radionuclide scintigraphy [66]

Staging (TNM)

Lung cancer tumours are currently staged according to the tumour-node-metastasis (TNM) classification system. Stage classification is based on the tumour-node-metastasis (TNM) staging system. The American Joint Committee on Cancer (AJCC) published the 1st edition of the Cancer Staging Manual in 1977. A revision of the TNM classification was implemented in 2018 using IASLC database which collects information on 94,708 cases with lung cancer, treated by all modalities of care between 1999 and 2010 [67].

In the VIII edition some changes were made for the T parameter. This parameter describes the location of the primary tumour, the size, and the degree of invasion of

nearby tissue. The T stages range from 1 to 4 where T1 tumors have a maximum size of 3 cm, T2 dimensions between 3 and 5 cm T3 dimensions between 5 cm and 7 cm and T4 tumors have dimensions greater than 7 cm in the largest diameter. Stages T1 and T2 are further divided into T1a / T1b and T2a / T2b. How much a primary tumor cannot be evaluated or that cannot be detected either with normal imaging techniques or with bronchoscopy but whose presence has been confirmed by the finding of neoplastic cells in the cytological examination of the sputum or in bronchial washings (BAL) is identified as Tx, while if not found, T0 is used to indicate stage T. Cancer in situ is designated Tis.

Parameter N describes the involvement of regional lymph node stations. Intrathoracic, scalene and supraclavicular lymph nodes are considered regional.

Depending on which lymph nodes are involved, 5 subgroups are identified; the N ranges from 0 to 3 where N0 means that there are no lymph nodes involved. N1 indicates the presence of metastases in one or more ipsilateral intrapulmonary and/or peri bronchial and/or hilar lymph nodes, including involvement by direct extension, N2 indicates the presence of metastases in one or more ipsilateral mediastinal and/or subcarinal lymph nodes, including "skip metastasis" without N1 involvement or associated with N1 disease. Finally, N3 indicates the presence of metastases in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene and ipsilateral or contralateral supraclavicular lymph nodes. Once again the use of the X instead of a number indicates the impossibility of evaluation, in this case of the regional lymph nodes. Growth of a tumor outside extraregional (extrathoracic) is classified as having distant metastases.

Distant metastases are evaluated with the parameter M, where M0 indicates the absence of distant metastases, while M1 indicates the presence. M1 is further subdivided, where M1a

If there are one or more separate tumor nodules in a lobe contralateral to the primary tumor, or if pleural or pericardial nodules are present, or if pleural or pericardial effusion of a malignant nature is present, M1b if the presence of a single extrathoracic metastasis is confirmed in a single organ or the involvement of a single non-loco-regional lymph node, M1c if multiple extra thoracic metastases are found in one or more organs [68].

Table 1 summarizes the TNM classification according to eighth edition.

Primary tumour (T)
- T1 – Tumour ≤3 cm in diameter, surrounded by lung or visceral pleura, without invasion more proximal than lobar bronchus.
- T1a – Tumour ≤2 cm in diameter.
- T1b – Tumour >2 cm but ≤3 cm in diameter.
- T2 – Tumour >3 cm but ≤7 cm in diameter, or tumour with any of the following features:
  - Involvement of the main bronchus ≥2 cm distal to the carina.
  - Invasion of visceral pleura.
  - Associated atelectasis or obstructive pneumonitis that does not involve the entire lung.
- T2a – Tumour ≤5 cm in diameter.
- T2b – Tumour >5 cm but ≤7 cm in diameter.
- T3 – Tumour >7 cm in diameter, or tumour with any of the following features:
  - Direct invasion of the chest wall, diaphragm, phrenic nerve.
  - Direct invasion of mediastinal pleura or parietal pericardium.
  - Associated atelectasis or obstructive pneumonitis of the entire lung.
  - Tumour within the main bronchus <2 cm to the carina, without involvement of the carina.
  - Satellite tumour nodules in the same lobe.
- T4 – Tumour of any size that has any of the following features:
  - Invasion of the mediastinum.
  - Invasion the heart or great vessels.
  - Invasion of the trachea, oesophagus or recurrent laryngeal nerve.
  - Invasion of a vertebral body or carina.
  - Separate tumour nodules in a different ipsilateral lobe.

Regional lymph nodes (N)
- N0 – No regional lymph node metastasis.
- N1 – Involvement of ipsilateral hilar or peri-bronchial nodes.
- N2 – Involvement of ipsilateral mediastinal or subcarinal nodes.
- N3 – Involvement of contralateral mediastinal or hilar nodes, OR involvement of ipsilateral/contralateral scalene or supraclavicular nodes.

Distant Metastasis (M)
- M0 – No distant metastasis.
- M1 – Distant metastasis present.
- M1a – Separate tumour nodule(s) in a contralateral lobe or tumour with pleural nodules or malignant pleural/ pericardial effusion.
- M1b – Distant metastases.

Table 1- TNM staging of NSCLC 8th edition[69].

After assigning the TNM parameters, it is possible to proceed with the classification in stages, from stage 0 to stage IV, where stages I-III are divided into groups a and b (see Table 2).

Stage	TNM 7th edition	cTNM 8th edition
I	T1N0	T1T2-N0N1
II	T2N0	T3-N0N2; T1T2-N2
III	T3-N0N1; T1T2-N1	$T4N_{any}$; TxN3
IV	IVa: T4a-N0N2c; T1T3-N0N2c IVb: $T4bN_{any}$; T_{any} N3 IVc: $T_{any}N_{any}M1$	$T_{any}N_{any}M1$

Table 2 -Stage Grouping- cTNM subsets 8th edition[69]

Staging is generally divided into clinical staging (cTNM), pathological staging (pTNM), clinical staging after neo-adjuvant therapy (ycTNM) pathological staging after neo-adjuvant therapy (ypTNM), based on the timing and assessments leading to staging.

RECIST

The Response Evaluation Criteria In Solid Tumors (RECIST) criteria are used to assess the response of solid tumors after therapy.

In the 1970s the World Health Organization (WHO) defined the WHO criteria, based on objective anatomical modification of the tumor to evaluate the response to anti-tumor

therapies, accepted throughout the 1980s. However, in the nineties the need arose to integrate the data deriving from new equipment such as CT and MRI to the classification that already existed, which led to the birth of a global collaboration. From this collaboration in February 2000 the classification "Response Evaluation Criteria in Solid Tumors" (RECIST) was born, introduced and published in the "Journal of the National Cancer Institute"[70].

The RECIST criteria are based on the "target lesions" and "non-target lesions", furthermore they have the concepts of number of evaluable lesions, lesion size, type of lesions and minimum measurable size, they laid the basis for a defined lesion model in time. They are based on the definition of the response by setting 4 categories (complete response, partial response, stable disease, and disease progression).

Tumor measurement is an objective method of assessing response to therapy and may be associated with regression or progression, which are the endpoints of many anti-neoplastic therapy trials.

In January 2009, processing a series of about 6,500 patients and 18,000 target lesions, the "RECIST Working Group" proposes the revision of the RECIST guidelines (renamed RECIST 1.1 criteria)[71].

However, RECIST criteria only consider the size of the tumors, but often this does not always correspond to a real response to therapy, the physiological and functional characteristics of the neoplastic tissue must also be taken into consideration in order to evaluate the response to cancer therapy in a standardized way. In recent decades, introduction of immunotherapy has made it necessary to revise the RECIST criteria.

In treatment with immunotherapy, the response time can be lengthened, and there is often an initial pseudo-progression[72].

The atypical responses seen in patients treated with immunotherapy will benefit from a reduction in tumor size following a clinically significant reduction in tumor burden (pseudoprogression) or an initial reduction in tumor size with the appearance of new lesions that subsequently regress[73]. To account for atypical tumor responses, several modified response criteria, called iRECIST, have been proposed [74] .

Table 3- Comparison of RECIST 1.1 and iRECIST[75]

NSCLC Treatment Approaches

	RECIST 1.1	iRECIST
Definitions of measurable and non-measurable disease; numbers and site of target disease	Measurable lesions are ≥10 mm in diameter (≥15 mm for nodal lesions); maximum of five lesions (two per organ); all other disease is considered non-target (must be ≥10 mm in short axis for nodal disease)	No change from RECIST 1.1; however, new lesions are assessed as per RECIST 1.1 but are recorded separately on the case report form (but not included in the sum of lesions for target lesions identified at baseline)
Complete response, partial response, or stable disease	Cannot have met criteria for progression before complete response, partial response, or stable disease	Can have had iUPD (one or more instances), but not iCPD, before iCR, iPR, or iSD
Confirmation of complete response or partial response	Only required for non-randomised trials	As per RECIST 1.1
Confirmation of stable disease	Not required	As per RECIST 1.1
New lesions	Result in progression; recorded but not measured	Results in iUPD but iCPD is only assigned on the basis of this category if at next assessment additional new lesions appear or an increase in size of new lesions is seen (≥5 mm for sum of new lesion target or any increase in new lesion non-target); the appearance of new lesions when none have previously been recorded, can also confirm iCPD
Independent blinded review and central collection of scans	Recommended in some circumstances—eg, in some trials with progression-based endpoints planned for marketing approval	Collection of scans (but not independent review) recommended for all trials
Confirmation of progression	Not required (unless equivocal)	Required
Consideration of clinical status	Not included in assessment	Clinical stability is considered when deciding whether treatment is continued after iUPD

"i" indicates immune responses assigned using iRECIST. RECIST=Response Evaluation Criteria in Solid Tumours. iUPD=unconfirmed progression. iCPD=confirmed progression. iCR=complete response. iPR=partial response. iSD=stable disease.

Type of therapy to be implemented is chosen on the basis of the characteristics of the tumor (stage of the disease, histological subtype and biomolecular characteristics of the disease) and all the characteristics of the patient (clinical conditions, presence of comorbidities). Therapeutic approaches in NSCLC include surgery, radiotherapy,

chemotherapy, immunotherapy, alone or in combination modality targeting therapy. Surgical resection with intent is recommended for medically fit patients with early stages of NSCLC (Stage I, Stage II and Stage III) and is associated with platinum-based or neoadjuvant especially for stages II-IIIA with a relapse rate and high toxicity. A valid alternative is stereotaxic radiotherapy (SBRT) which is characterized by the administration of biologically very high doses in one or a few fractions and allows for better results than the conventional one. At diagnosis includes 30% of patients with NSCLC are already in locally advanced disease (T3-T4, N2-N3, stage IIIA-C) . Most patients with stage III NSCLC are nonsurgical candidates and the current standard of care is chemoradiotherapy.

Targeted therapy has improved clinical outcomes in a percentage of NSCLC patients with advanced disease, further the indication is to perform molecular tests on panel in order to identify possible tumor biomarkers. Genetic alteration-targeting tyrosine kinase inhibitors EGFR, ALK, ROS1, RET, BRAF V600E, MET Exon 14, and NTRK are now approved for the treatment of several NSCLC patient subtypes.

Another important strategy is immunotherapy, which uses immune checkpoints or a protein molecule expressed on the membrane surface of T lymphocytes and plays a key role in maintaining homeostasis of the immune system as it guarantees tolerance towards self and antigens. they limit autoimmunity. The purpose of monoclonal individuals belonging to the class of immune checkpoint inhibitors is precisely to block these signaling pathways and reactivate the antitumor immune response. Currently the drugs authorized by the FDA and AIFA are Ipilimumab (CTLA-4 inhibitor), Nivolumab and pembrolizumab (PD-1 inhibitors), Atezolizumab, Durvalumab and Avelumab (inhibitors PD-L1). In the following two images (Figure X and Figure X) the AIOM guidelines for the treatment of patients with localized, locally advanced or metastatic NSCLC have been adapted and schematized [76].

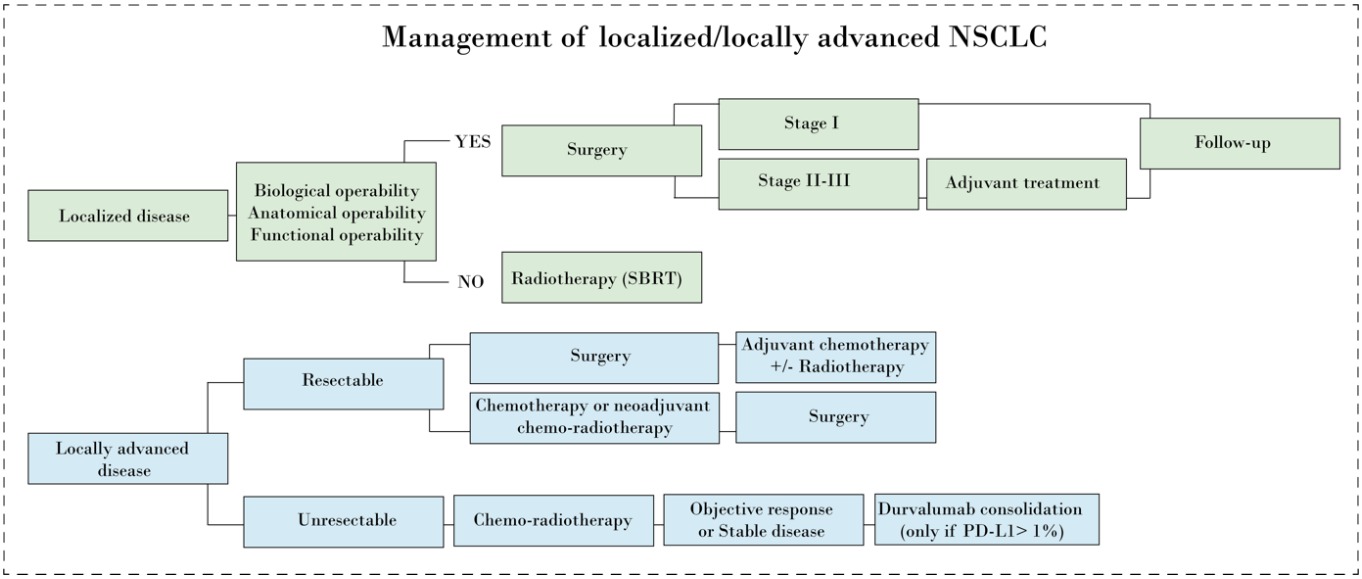


Figure 12 -Management of localized/locally advanced NSCLC. Adaptation of figure in AIOM 2020 guidelines[76]

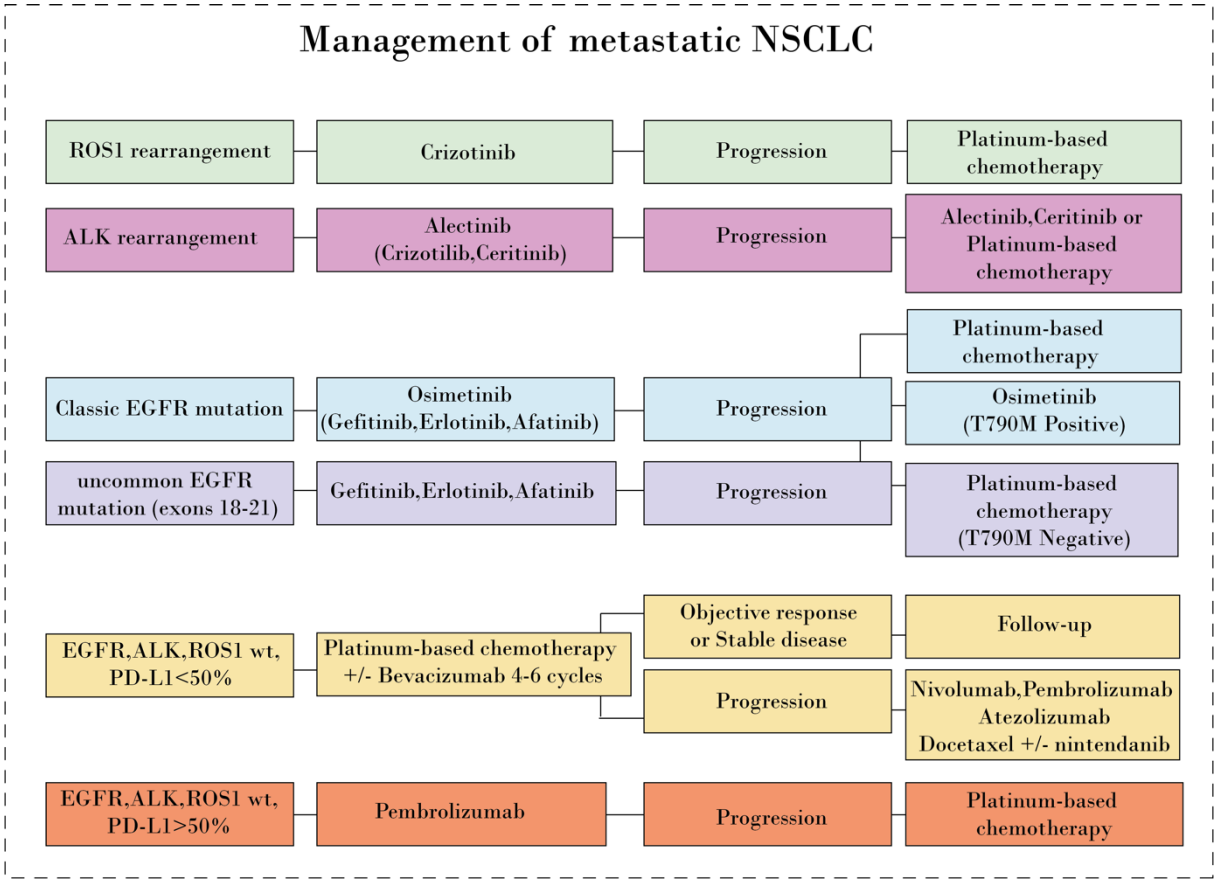


Figure 13 -Management of metastatic NSCLC. Adaptation of figure in AIOM 2020 guidelines[76]

Biomarkers in Lung Cancer

Recent advances in molecular characterization of lung cancer has greatly changed our understanding of cell signaling pathways that control cell survival have identified genetic and regulatory mutations that suppress cell death, promote cell division and induce tumorigenesis, becoming an essential component of pathological diagnosis and oncological therapeutic decisions.

These new discoveries have led to the identification of well-defined subgroups of patients with an "oncogene addicted" tumor or with molecular characterization of the different stages of the disease and some differences in terms of clinical and demographic

characteristics identify subgroups of patients benefiting from personalized molecular therapies.[77]

Biomarker was defined as “an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers " in according to BEST (Biomarkers, EndpointS, and other Tools), developed in 2016 by FDA (Food and Drugs Administration) and NIH (National Institute of Health)[78].

Many biomarkers have been studied over the years in the field of lung cancer, most of which belong to the category of predictive or prognostic biomarkers that represent optimal candidates for developing a targeted therapy.

The first candidate was the epidermal growth factor receptor (EGFR), it is a transmembrane receptor tyrosine kinase expressed in some normal epithelial, neurogenic and mesenchymal tissues [79]. Activation of this receptor involves activation of various intracellular pathways, including phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and RAS/RAF/mitogen activated protein kinase (MAPK) pathways, leading to cell proliferation, metastasis and the prevention of apoptosis [80]. EGFR overexpression is found in 16% of diagnoses and EGFR point mutation is found in 10-15% of diagnoses and has been associated with a poor prognosis [81].

Other biomarkers discovered are homologous viral oncogene of Kirsten's rat sarcoma (KRAS), tyrosine kinase of anaplastic lymphoma kinase (ALK) and ROS Proto-Oncogene 1 (ROS1). Activating alteration of these oncogenes occur in 25%, 2% and 1% of patients with NSCLC, respectively [8283–85].

Unfortunately, relapse after targeted therapy against these biomarkers is almost inevitable and tends to occur after about one year of EGFR therapy and after 8 and 19 months with ALK and ROS1 target therapy, respectively. The EGFR mutation identified as

T790M is found in 50-60% of progressive patients, making first generation tyrosine kinases ineffective[86]. Even the onset of mutations downstream of the ALK and ROS1 pathway can make these therapies ineffective, such as MET amplification or constitutive activation of PIK3CA[87].

In order to identify new biomarkers research went on to analyze less common but potentially druggable alterations such as BRAF (2-4%), ERBB2 (1-2%), NTRK (0.5-1%) amplifications or mutations of MET (overall 2-4%) and RET (1-2%), and mutations in G12C KRAS hotspots (14%) [88].

In parallel with drug development, diagnostics has evolved by implementing classical methods such as FISH, IHC and RT PCR with NGS methods. The new NGS technology is able to analyze hundreds of gene mutations in a single analysis quickly and with high accuracy of the results. This analysis allows to obtain a genomic profiling of the tumor and helps to optimize the diagnosis and the therapeutic path of the patient by identifying all potential drug targets with a single examination. A greater ability to identify predictive biomarkers of potential therapeutic efficacy allows understanding the dynamics of disease progression and response to treatment, allowing for more optimal monitoring.

Furthermore, increasing complexity of the "Oncogene addicted" panorama in the various types of tumors including NSCLC, tools such as the ESCAT scale (ESMO Scale for Clinical Actionability of molecular Targets) have been developed. This scale is applied in clinical practice only in cases of evident clinical benefits (actionability of the different targets) and economic sustainability compared to alternative methods such as Real Time PCR or; in non-small cell lung cancer (NSCLC), prostate cancer, ovarian cancer and cholangiocarcinoma [89,90].

The new ESCAT system classifies tumor DNA alterations based on their relevance as markers for selecting patients for targeted treatment, based on the strength of the clinical evidence that supports them, for example if a patient has two mutations one of level I and the other level II, the targeted therapy to choose is level I as it has greater clinical value, risk and economic sustainability.

The ESCAT scale provides for a classification of druggable genomic alterations in four levels:

- ESCAT I: genomic alterations with predictive value validated in clinical studies.
- ESCAT II: genomic alterations associated with drug response in phase I/ II studies or in retrospective analyzes of randomized studies.
- ESCAT III: genomic alterations validated in another tumor, but not in the specific one to be treated.
- ESCAT IV: genomic alterations that can be hypothetically attacked on the basis of preclinical data.

Based on ESCAT, genomic alterations in NSCLC tumor patients include EGFR mutation, ALK rearrangement, BRAFV600E mutation, RET rearrangement, ROS1 rearrangement, NTRK rearrangement, and $MET^{Ex14Skipping}$ mutation. Table 4 summarizes the main alterations and the associated ESCAT level.

Gene	Alteration	Prevalence	ESCAT
EGFR	Common mutations (Del19,L858R)	15%(50%-60% Asian)	IA

	Acquired T790M exon 20	60% of EGFR mutations NSCLC	IA
	Uncommon EGFR mutations (G19Xin exon 18,L861Q in exon 21, S768I in exon 20)	10%	IB
	Exon 20 insertions	2%	IIB
ALK	Fusions (mutation as mechanism of resistance)	5%	IA
MET	Mutations ex 14 skipping	3%	IB
	Focal amplifications (acquired resistance of EGFR TKI in EGFR- mutant tumours)	3%	IIB
$BRAF^{V600E}$	Mutations	2%	IB
ROS1	Fusions (mutations as mechanism of resistance)	1-2%	IB
NTRK	Fusions	0,23-3%	IC
$KRAS^{G12C}$	Mutations	12%	IIB
RET	Fusions	1-2%	IC
ERBB2	Hotspot mutations, Amplifications	2-5%	IIB
BRACA ½	Mutations	1-2%	IIIA
PIK3CA	Hotspot mutations	1,2-7%	IIIA
NRG1	Fusions	1,7%	IIIB

Table 4 - List of genomic alterations level I/II/III according to ESCAT in advanced non-squamous non-small-cell lung cancer (NSCLC)[89].

Progress on cancer genomics by NGS

Lung tumor heterogeneity was recently confirmed in an exome sequencing study of 31 non-small cell lung cancer (NSCLC) identifying 727 mutated genes not previously reported in the literature or in the COSMIC database [91]. The use of biomarkers in the clinic to determine patient prognosis and response to therapy are valuable tools that can increase patient survival and reduce toxicity from ineffective treatments.

For many years, standard techniques such as FISH, IHC have been used in routine practice for the analysis of mutations targeted at the hotspot and used in a highly selective approach to testing a single gene.

These techniques were programmed following a pragmatic algorithm that was initially set up and subsequently modified with the approval of specific target therapies.

Sequential testing starting in 2009 included a first analysis of EGFR and KRAS as gefitinib, an anti-EGFR molecularly targeted drug, was approved. In the case of EGFR and KRAS wild type patients, the evaluation proceeded with the sequential mutational analysis of the alterations of all the other genes; *BRAF*, *RET*, *NTRK*, HER2, KRAS and *MET*.

After European Medicines Agency (EMA) approval of crizotinib on 23 October 2012, the algorithm was expanded to include ALK determination for EGFR and wild type KRAS patients.

After EMA approval of crizotinib for ROS1 on 21 July 2016, this genetic test was added for wild type ALK and finally in case of wild type KRAS, EGFR, ALK and ROS1 tumors were

analyzed. BRAF, MET, HER2 and RET depending on the availability of clinical studies with experimental agents aimed at the specific alteration of the gene[92].

The need to perform multiple 8-10 gene sequential analyzes has been a major challenge for laboratories, as this approach is costly, time-consuming and requires significant amounts of tumor nucleic acid. In many cases, the amount of tumor tissue available is less than that required to complete multiple molecular tests, in addition to FISH and IHC [93].

The expansion of available approved targeted drugs has caused a growing demand for multigen testing for more efficient simultaneous detection of targeted alterations.

In this new reality, next-generation sequencing (NGS) has recently been recommended by the 2020 European Society for Medical Oncology (ESMO) guidelines to perform a comprehensive molecular characterization in patients with metastatic lung adenocarcinoma, prostate cancer, ovarian cancer, or cholangiocarcinoma[89].

The NGS panels currently used in clinical practice are of two types:

• Hybrid-capture where the target sequences are captured using biotinylated probes.

• Amplicon-Based Methods where the target sequences are first amplified by PCR with specific known primers.

The advantage of the first technique is to identify also rearrangements in which the fusion partner is not known, but unlike the amplicon technique in which results are obtained even starting from a small quantity of nucleic acid (up to 10 ng per reaction), the technique does not allow to obtain results without a higher amount of extracted DNA[94]. Consequently, limit of the NGS test depends on the percentage of available tumor cells within the sample, NGS is able of detecting single nucleotide variants in samples with

tumor cellularity >20%, while it decreases the probability of identifying molecular alterations in samples with tumor cellularity between 10 and 20%[95].

To overcome the problem, a liquid biopsy was developed which is based on the analysis of venous sampling, making the examination less invasive than traditional biopsies[96].

There are currently three types of circulating biomarkers that can be detected in liquid biopsy: circulating tumor cells (CTC), circulating tumor DNA (ctDNA) and exosome. Several studies have demonstrated a high concordance between NGS on ctDNA and matched tissue, validating the methodology[97,98].

Moreover it would seem that the digital polymerase chain reaction (dPCR) technique is a very sensitive method for ctDNA. Several studies have shown that dPCR can also be used for quantitative measurements of the copy number of the T790M mutant in plasma cfDNA to predict treatment response and survival outcomes in NCSLC patients[99]. The Nanostring nCounter technique also represents a genomic platform based on a color-coded dual probe system that allows direct counting of specific mRNA molecules in a single reaction potentially useful for the detection of gene fusions in clinical practice and also suitable in situations of analysis of degraded clinical samples[100,101].

Another interesting point of discussion consists in using the multigene panels to analyze also markers that are not in clinical practice today but that would allow to guide patients towards therapeutic studies [93].

The advancement in the clinical and pharmacological field nowadays determines a growing need to identify clinically relevant markers for this reason it is necessary that the technologies used for the analysis also improve together with the medical-clinical needs.

Two techniques are currently being tested; The Nanopore allows you to sequence a single nucleic acid molecule (DNA or RNA) without PCR pre-amplification as it records an electrical signal as the DNA molecule passes. Another experimental technique is the integrated non-overlapping read sequencing system (NOIR-SS) is a sequencing method that uses molecular barcodes, such technology has been successfully used to identify the p.L858R mutation of EGFR in plasma of patients with lung adenocarcinoma [102].

NGS allows maximizing the identification of clinically relevant genomic alterations using a limited amount of tissue, thus reducing time and costs. The costs per sample of NGS have drastically decreased over time and allow for a broader molecular characterization for more accurate patient stratification[103]. According to, NGS allows the identification of co-mutations and rare mutations, allowing you to have a complete screenshot of the patient's molecular profile. Thanks to the introduction of next-generation sequencing, we can now easily identify pathology markers and even a large number of NSCLC-related genes[104].

Cancer immunoediting

Immunotherapy is an innovative treatment against cancer. The original idea that the immune system was able to protect the organism from tumor genesis, through a complex interaction between cancer and the immune system has been known for many decades. The theory of immunosurveillance was definitively confirmed in the 1990s, thanks to the discoveries of the role of IFN-γ, perforin and the use of mouse strains deficient in T, B and T NK lymphocytes (but with intact mechanisms of DNA repair); in this case the

experimental tests confirmed the role of lymphocytes as cells able of protecting the host from the appearance of both chemically induced and spontaneous tumors.

This suggests that tumor evolution in hosts with competent immune systems is associated with the selection of poorly immunogenic cells and/or ability to escape the immune-mediated effector mechanisms that would allow their rejection. These observations, therefore, indicate that the immune system plays a very complex role through immunosurveillance. Accordingly, the concept of immunosurveillance, albeit correct, should be expanded to define the dual role of the immune system, no longer only protective, but also "tumor sculpting"[106].

In 2002 a new theory was proposed by GP Dunn, LJ Old and RD Schreiber under the name of tumor immunoediting. "Cancer immunoediting", a dynamic process describing the influence of the immune system on tumor progression, proposes three distinct phases of immune surveillance: elimination, balance, and escape[107].

- Elimination, transformed tumor cells are successfully destroyed by the host's immune system;
- Balance, in which cancer cells; The host's immune system is unable to eliminate eliminate cancer cells but can adequately limit their growth, through a selection process operated by T lymphocytes, they become resistant to the control of the immune system and bring the cancer cells to a period of dormancy.

- Escape or evasion, cancer cells after having had several genetic changes can evade the immune system, in which diseased cells spread uncontrollably, resulting in clinically detectable neoplasms.

This concept provides in summary a dynamic vision of the complex interactions between surveillance and cancer for which cancer cells, subjected to the selective pressure of the immune system, tend to be characterized in an increasingly aggressive way, managing at the end of the process to escape control[108].

Immune biomarkers in NSCLC

Under normal physiological conditions, the immune checkpoint molecules regulate the activation of T cells to generate an effective immune response, helping to maintain peripheral tolerance towards self-molecules and thus reducing excessive inflammation or autoimmunity.

Bretscher et al laid the foundation for the concept of "two signals" of lymphocyte activation which investigates the mechanisms of interaction between co-signaling molecules on the surface of T cells and antigen-presenting cells (APCs) in order to activate the naive T cell. Furthermore, this model explains both the discrimination of self from non-self and immunization concerning induction of tolerance for B cell responses[109] .

Through these studies, the importance of co-signaling molecules in promoting or inhibiting the immune response was understood.

There are currently a large number of co-stimulating T-cell molecules with distinct and overlapping functions. One of these is the B7-1/B7-2: CD28/cytotoxic T-cell-associated

antigen 4 (CTLA-4) pathway plays a key role in regulating T cell activation and tolerance [110,111].

Two inhibitory immune checkpoint receptors have been extensively studied: antigen 4 associated with cytotoxic T lymphocytes (CTLA-4) and programmed cell death protein 1 (PD-1). Despite acting on distinct targets, both CTLA-4 and PD-1 belong to the B7 receptor family and are expressed on the surface of T cells[112,113].

CTLA-4, a CD28 homolog, competitively binds B7-1 B7-2 on APCs with high affinity leading to partial activation (anergy) of the T cell, unlike complete activation driven by the CD28-B7 bond. The co-stimulation brought about by the binding between CD28-B7 regulates the threshold for the activation of T cells, determines a greater proliferation, determines a massive production of cytokines and their receptors also causes an increase in the expression of proteins involved in the progression of the cell cycle. Blocking the link between CTLA-4-B7 leads to a more aggressive and intense immune response[114].

The other important immune checkpoint is represented by the programmed cell death protein 1 (PD-1), discovered in 1992 by Honjo, while studying the mechanisms of T cell death[115]. The main activity of PD-1 is to inhibit the activation of T cells during long-term exposure to antigen, as occurs in chronic viral infections and tumors. Under normal physiological conditions, it has been shown that PD-1 binds to PD-L1 or PD-L2 expressed on the host's APC cells, preventing hyperactivation and maintaining self-tolerance[116,117]. However, many solid tumors express the PD-L1 receptor by exploiting this mechanism to evade the immune system by generating an immunosuppressive effect. Tumor selectivity

of PD-1 or PD-L1 provides a great opportunity for patient selection based on tumor markers.

Upregulation of these immune checkpoint proteins leads to T cell inactivation and inefficient killing of tumor cells mediated by T cells. These immune checkpoints have become targets for a new type of drug called immune checkpoint inhibitors.

Immune checkpoint inhibitors

The use of immune checkpoint inhibitors (ICI) has become one of the most promising approaches in the field of cancer therapy particularly in the treatment of advanced non-small-cell lung cancer (NSCLC).

Monotherapy

The standard treatment for advanced NSCLC patients who have progressed after chemotherapy has been docetaxel monotherapy (DOC) for many years. Several randomized controlled trials were conducted to evaluate the efficacy of ICI (nivolumab, pembrolizumab, atezolizumab) compared to DOC and proved to be superior in terms of median OS (about 3 months longer) and relation to toxicity[118,119].

Similar results in particular survival benefit and reduced toxicity were observed for pembrolizumab compared to docetaxel in the second-line treatment of PD-L1-positive NSCLC in study KEYNOTE 010 and the POPLAR and OAK studies for atezolizumab[120–122]. These important results have laid the foundation for immunotherapy to become the new standard treatment after first-line chemotherapy. However, ICIs have been shown to be more effective in patients with NSCLC that do not harbor oncogenic gene alterations

(CheckMate012)[123]. Subsequently, we wanted to investigate the effectiveness of ICI also on the front line, in 2016 the FDA approved pembrolizumab in NSCLC patients with metastases and with a PD-L1 expression> 50% on tumor tissue, based on data from study KEYNOTE 024 phase III trial[124]. Similar studies with slightly different inclusion and exclusion criteria have also been re-proposed with Atezolizumab (IMpower110) and Nivolumab[125]. However, for the latter, there were no benefits in terms of PFS or OS for the nivolumab group and the control group. Consequently, nivolumab was only approved for the treatment of NSCLC in the second-line setting.

Combination therapy

To increase the response rate, combinations of PD-1 or PD-L1 blockade with other treatments, such as chemotherapy, targeted therapies and other ICIs, and impressive improvements in first-line treatments were investigated.

For example, Pembrolizumab plus platinum-based chemotherapy has been shown to be more effective than front-line chemotherapy alone and has led to speeding up the approval of first-line immunotherapy for patients with NSCLC. Another study showed how the treatment of ICI plus PFS and overall survivals OS chemotherapies was associated with longer and a comparable risk of adverse events compared to bevacizumab plus chemotherapy[126].

The combination of ICIs targeting two or more immune checkpoint molecules also brings synergies into clinical practice. In the phase III study CheckMate 227, the combination of nivolumab and the anti-CTLA-4 antibody ipilimumab was studied in 793 NSCLC patients with untreated IV status. Results showed that nivolumab PFS in combination with

ipilimumab and chemotherapy alone was 7.2 months vs.5.5 months (p <0.001), mOS was 17.1 months vs 14.9 months (p = 0.007), and was independent of the level of PD-L1 expression[127]. Additionally, the ORR (45.3%) of nivolumab in combination with ipilimumab in first-line NSCLC treatment was superior to that of chemotherapy alone (26.9%) with comparable adverse events to chemotherapy in advanced NSCLC. Furthermore, for patients with chemotherapy contraindications, the combination of two ICI therapies has greater advantages over chemotherapy combined with ICI therapy.

Contrary to clinical studies such as the baseline study 1b TATTON has shown how the combination of TKI EGFR targeted with ICI led to an increase in irAE such as interstitial pneumonia[128].

AIM OF THESIS

The overall aim of this Ph.D. project was to explore the relevance of NGS sequencing and accurate histological subtyping to identify rare molecular and/or pathological subtypes(molecular and histological) of advanced NSCLC which may allow a personalized effective treatment through the enrollment into clinical trials.

More specifically, the aims of this thesis were:

- The first part of the project aimed to analyze the molecular profile of patients belonging to S.Orsola-MalpighiUniversity Hospital, using multi-gene NGS as a diagnostic tool to identify subpopulations of patients with potentially actionable rare mutations Allowing the enrollment of patients into clinical trials with novel targeted agents
- The second part of the project aimed to activate a clinical study for the treatment with immunotherapy of patients with rare lung cancer histologies.
- The third part of project aimed to activate a clinical study for patients with a rare molecular alteration (RET rearrangement) to assess activity of a specific molecularly targeted therapy.

Molecular profiling of NSCLC-A

Introduction

Lung cancer is one of the most common causes of cancer death worldwide in both males and females and is the second most common cancer in males and third in females. Recently, the discovery of generation sequencing (NGS), along with the next generation of new targeted therapies, has dramatically revolutionized the molecular understanding of cancer. Pulmonary adenocarcinoma heterogeneity was recently confirmed in an exome sequencing study of 31 NSCLC identifying 727 mutated genes not previously reported in the literature or in the COSMIC2 database. Lung cancers represent one of the main health costs in oncology. In the latter, numerous targeted anti-tumor therapies have been developed for lung tumors directed against specific molecular alterations of neoplastic cells. Starting from 2009, anti-EGFR, ALK, and ROS1 drugs have progressively

entered the market with evident efficacy in young, non-smoking patients with lung tumors. Numerous other drugs are undergoing clinical trials directed against alterations of the BRAF, HER2, MET, RET, KRAS, and NTRK genes, always in patients with lung tumors. Consequently, the application of NGS is a powerful tool for discovering both druggable and new mutations to identify the best therapeutic strategy or select these patients for personalized therapies currently undergoing clinical trials.

Methods

Study design and objectives

This project is a prospective monocentric cohort study conducted at Sant' Orsola-Malpighi University Hospital in Bologna by the Medical Oncology unit with the participation of the Pathology and molecular pathology units. The study involved patients with NSCLC, whose tumor tissues were analyzed at our institution from January 2019 to September 2021, to identify and characterize the main gene mutations using multi-gene NGS technology. This project allowed us to evaluate molecular profiles in patients with advanced NSCLC to choose the most appropriate treatment.

Patients

We analyzed a prospective cohort of 1408 patients with advanced NSCLC-A treated at the Sant' Orsola-Malpighi University Hospital. Inclusion criteria were histological diagnosis of A-NSCLC with tumor tissue available for biomolecular analysis and adequate (at least 20% enrichment in neoplastic cells and at least 20 ng of DNA and 50 ng of RNA extracted), age higher or equal to 18 years. Both histological and cytological specimens were included.

Molecular analysis

Analysis was performed with a massive parallel NGS sequencing method.

The samples consisted of small formalin-fixed, paraffin-embedded biopsies or cytological smears obtained during bronchoscopy. NGS was performed starting from 10 µm thick serial sections or cytological smears encompassing the tumor area of interest with at least 50% tumor cell enrichment. After manually micro dissecting the slides, the RNA and DNA were isolated. Complementary DNA (cDNA) synthesis before library preparation for the RNA panel was performed using the SuperScript ™ VILO ™ cDNA synthesis kit (Thermo Fisher Scientific, 11754050). Libraries were prepared using the Oncomine AssayTM, 318 Solution (Thermo Fisher Scientific) assay using a total of 50 ng and 20 ng of RNA and DNA inputs, respectively, per sample. Sequencing was performed using the Ion PGMTM Hi-QTM View Sequencing Kit on the Ion Torrent S5 Personal Genome Machine (Thermo Fisher Scientific).

The Oncomine Focus Assay Gene Panel - Thermo Fisher Scientific, Carlsbad, CA (RUO Kit) 3 which can simultaneously provide information on mutations, fusions, and copy number variations of over 50 genes involved in therapy. Figure 14 summarizes hotspot mutations in 35 genes, amplification in 19 genes, and rearrangements in 23 genes.

Hotspot genes (35)				Copy number variants (19)			Fusion drivers (23)		
AKT1	*ERBB3*	*IDH2*	*MTOR*	*ALK*	*FGFR2*	*PIK3CA*	*ABL1*	*ETV4*	*PDGFRA*
ALK	*ERBB4*	*JAK1*	*NRAS*	*AR*	*FGFR3*		*AKT3*	*ETV5*	*PPARG*
AR	*ESR1*	*JAK2*	*PDGFRA*	*BRAF*	*FGFR4*		*ALK*	*FGFR1*	*RAF1*
BRAF	*FGFR2*	*JAK3*	*PIK3CA*	*CCND1*	*KIT*		*AXL*	*FGFR2*	*RET*
CDK4	*FGFR3*	*KIT*	*RAF1*	*CDK4*	*KRAS*		*BRAF*	*FGFR3*	*ROS1*
CTNNB1	*GNA11*	*KRAS*	*RET*	*CDK6*	*MET*		*EGFR*	*MET*	
DDR2	*GNAQ*	*MAP2K1*	*ROS1*	*EGFR*	*MYC*		*ERBB2*	*NTRK1*	
EGFR	*HRAS*	*MAP2K2*	*SMO*	*ERBB2*	*MYCN*		*ERG*	*NTRK2*	
ERBB2	*IDH1*	*MET*		*FGFR1*	*PDGFRA*		*ETV1*	*NTRK3*	

Figure 14- Oncomine Focus Assay gene list.

Outcomes

The data analysis was carried out with a descriptive method and compared with the cases present in the literature.

Results

1408 tissues were sequenced between January 2019 and September 2021. 799 patients, corresponding to 56.7%, were male and 609, (43.3%), were female. 33 samples showed < 20% enrichment in cancer cells or were not suitable for NGS sequencing and were therefore excluded from the analysis. The main biopsy sites were lung (50%) lymph nodes (29%) pleura (10%) liver (4%) bone (2%).

Overall, 1169 (85%) specimens harbored driver mutations in 8 cancer genes (EGFR, KRAS, ALK, ROS1, MET, BRAF, RET, and HER2), which are canonical driver mutations, while 17% were considered wild type (WT) as none of the genomic alterations covered by our panel was found. EGFR alterations were detected in 233 patients (16%). AKRAS mutation was found in 456 patients (33%). Patients with a rearrangement of the ALK gene or the ROS1 gene were found to be 39 (3%) and 14 (1%), respectively. There were 83 (6%) patients with MET mutation, 19 (1%) with MET amplification, 72 (5%) with BRAF mutation, 36 (3%) with RET rearrangement, and 47 (3%) with HER2 amplification (Figure 15).

In total, 27% of patients had at least one genomic alteration in the druggable genes according to the ESCAT I classification (EGFR mutation 11%, ALK rearrangement 3%, $BRAF^{V600E}$ mutation 5%, RET rearrangement 3%, ROS1 rearrangement 1%, NTRK rearrangements 0,2%, and $MET^{Ex14Skipping}$ mutation 3%).

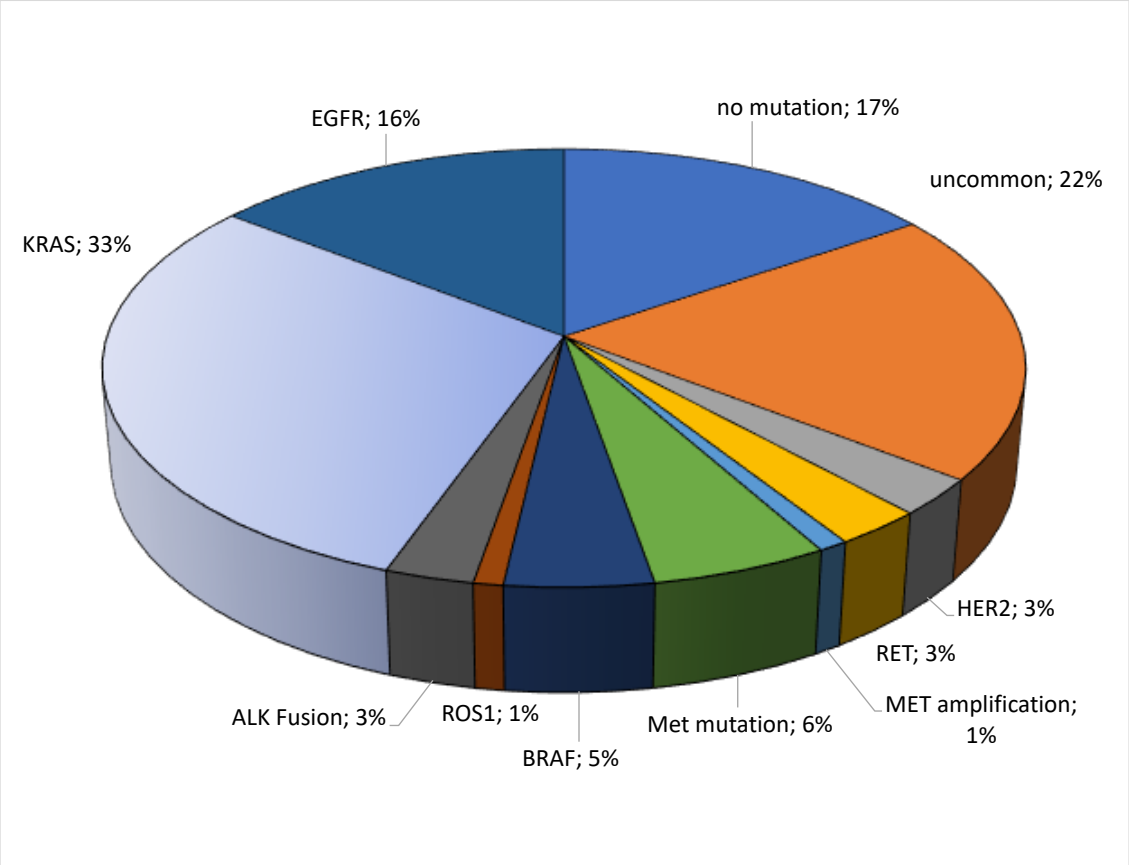


Figure 15- Characterization of molecular profiling NSCLC Cancer in IRCCS- S.Orsola Malpighi's population.

36 patients had uncommon alterations which are summarized in figure 16.

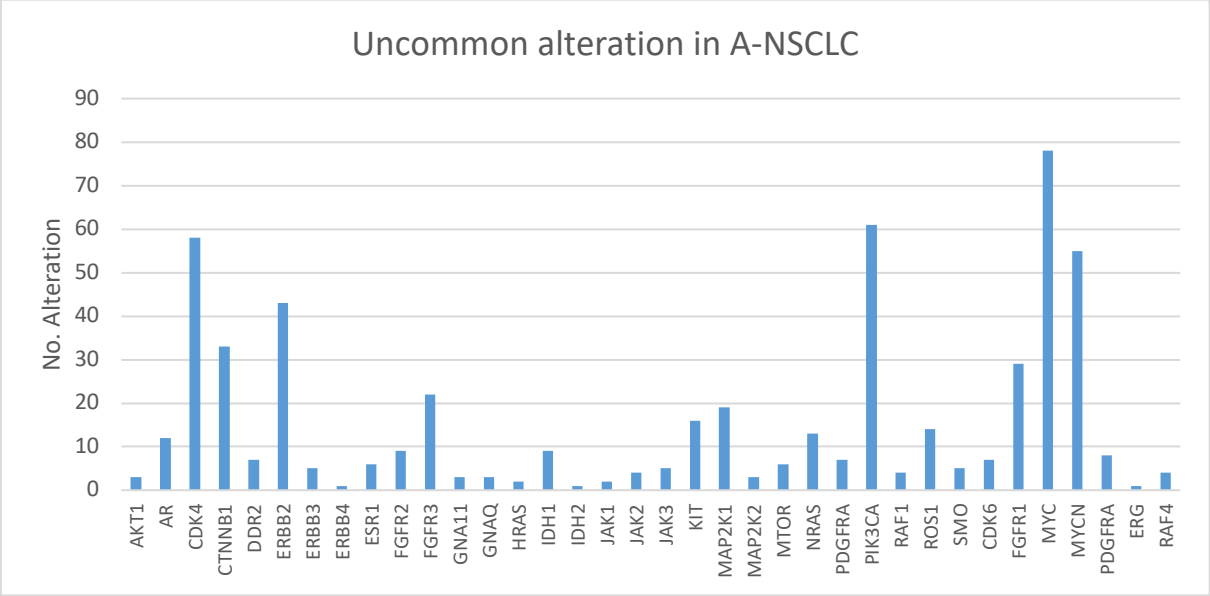


Figure 16 - Uncommon alteration of A-NSCLC

410 patients presented an actionable genetic rare variant for which targeted therapy is available. Indeed, molecular profile found in many patients harbored non-actionable variants with the possibility of recruitment in clinical trials according to international guidelines or options for off-label treatment. For example, 233 carried an EGFR mutation. Of those, exon 19 deletion and exon 21 L858R point mutation were the most common findings, comprising 33% and 25% of all mutations, respectively, while exon 18 accounted for 5% and exon 20-point mutation for 11%. (Figure 18).

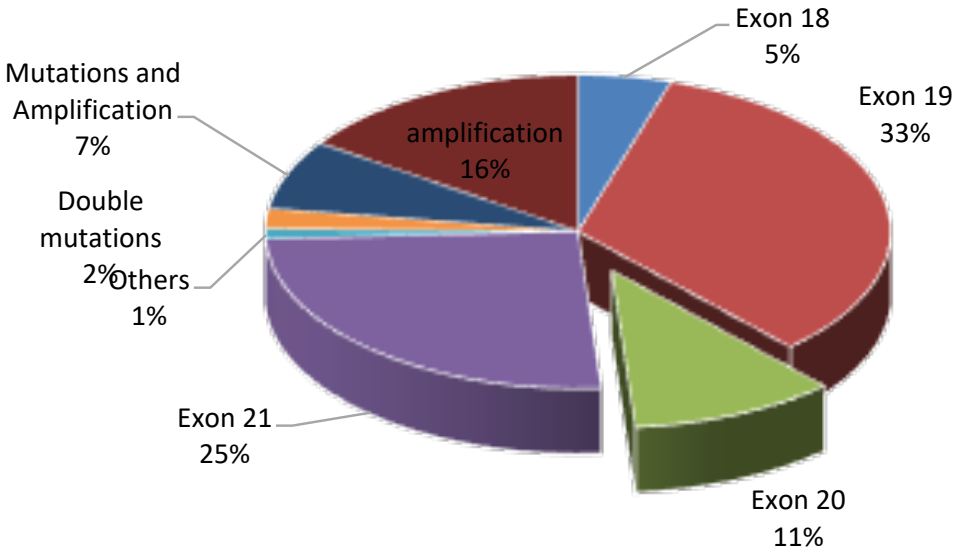


Figure 17-. Frequencies of different types of EGFR mutations

11% of subjects had an exon 20 mutation, of these 1 patient is currently enrolled in PAPILLION study, which involves treatment with Amivantamab, a bispecific antibody targeting EGFR and MET e[129].

KRAS was the most common molecular alteration with 456 patients mutated on 1375 analyzed (33%). The most common KRAS mutation was G12C (36%) followed by G12D (16%), G12V (18%) and G12A (8%) and other (19,3%) (Figure 19).

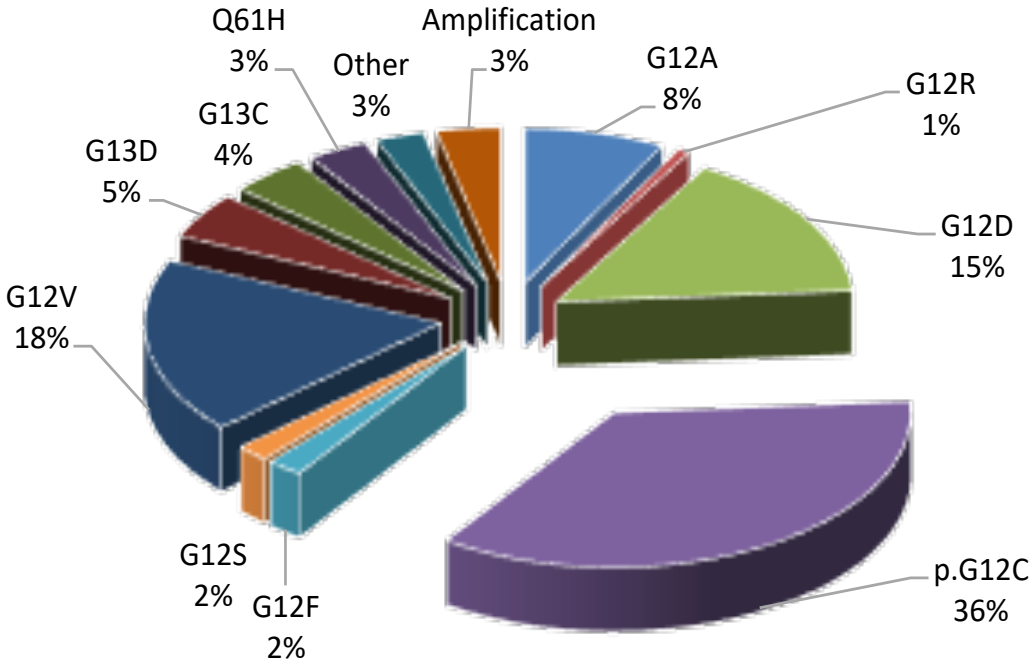


Figure 18-Frequencies of different types of Kras alterations.

KRAS G12C mutation was found in 36% of subjects with KRAS mutation. AMGEN clinical study "A phase 3 study comparing AMG 510 with Docetaxel in subjects with KRAS p G12C mutation NSCLC" is available at our hospital. Currently 5 patients have been enrolled, of which 2 entered treatment and 3 were screening failures. X other patients were treated with AMG510 within a compassionate use program

In our study the percentage of BRAF mutations was 5% of which 36% were V600E mutations. Clinical Trial ARRAY 818-202 "A Phase 2, Open-label Study of Encorafenib + Binimetinib in Patients with BRAFV600-mutant Non-small Cell Lung Cancer"[130,131] is available in our unit. Currently 7 patients have been enrolled and treated with a novel combination of anti-BRAF and anti-MEK agents.

Another molecular alterationspecifically found in NSCLC patients is the ALK fusion that, in our study, was found in 39 patients (3%). The detected primary ALK fusion identified was as EML4-ALK followed by KIF5-ALK.

Other molecular alterations have recently been found in patients with A-NSCLC, such as the translocation of RET, FGFR, NTRK 1-3 and MET exon14 skipping mutations.

We enrolled 5 RET-rearranged patients in J2G-MC-JZJC clinical trials assessing selective (Selpercatinib)[132–134] RET-inhibitors, 2 subject with FGFR mutation or translocations enrolment in a Phase 2, Open-Label, Single-Arm, Multicenter Study with Pemigatinib (FISHT-207).[134]

In study CINC280J12201, which evaluated the combination of Capmatinib plus Spartalizumab vs Capmatinib plus placebo in patients with mutations that skip exon 14 MET (3%), we enrolled two patients[135].

We found 0,2% of NTRK 1-3 mutations that could possibly be enrolled in the basket study LOXO_TRK-15002 which involves treatment with Larotrectinib [136].

Interestingly, 31% of the patients (423) had co-mutation such as PIK3CA, MYC, CD4K and CCD1 respectively 65%, 57%, 40% and 36%, which are summarize in Figure 17.

Biomarker	KRAS	EGFR	MET	RET	BRAF	HER2	ALK	No.Co-mutation	%
	456	233	102	36	72	47	67	423	
ALK	16	3	0	1	3	0		23	34%
AR	3	0	2	0	0	0	6	11	92%
BRAF	24	0	6	1		0	3	34	47%
CDK4	2	6	13	1	1	0	1	24	41%
CTNNB1	9	13	3	3	0	1	1	30	91%
HER2	5	3	3	0	0		0	11	26%
ERBB3	4	0	2	0	0	0	0	6	75%
ERBB4	0	0	1	0	0	0	0	1	50%
ESR1	4	0	2	0	0	0	0	6	67%
FGFR1	0	0	1	0	0	0	0	1	4%
FGFR2	5	0	1	0	0	0	0	6	67%
FGFR3	7	4	0	0	0	0	0	11	50%
GNAQ	1	0	0	0	0	0	0	1	33%
NRAS	7	0	0	0	0	0	0	7	54%
IDHI	5	0	0	0	0	0	0	5	50%
JAK	6	0	0	1	1	0	1	9	82%
KIT	1	0	3	0	1	1	0	6	38%
MAP2K1	9	0	0	0	0	0	1	10	53%
MET	27	18		2	0		2	49	48%
MTOR	3	2	0	0	1	0	0	6	100%
PIK3CA	21	11	7	0	2	0	0	41	67%
RAF	1	0	0	0	0	1	0	2	50%
RET	7	0	0		0	0	0	7	19%
ROS1	1	0	0	0	0	0	0	1	7%
SMO	1	0	0	1	1	0	0	3	60%
MYC	23	14	7	0	0	0	2	46	59%
MYCN	0	0	1	0	0	0	0	1	20%
CCND1	0	6	5	0	1	4	0	16	37%
CDK6	0	1	0	0	0	0	3	4	57%
DDR2	0	4	0	1	0	0	0	5	71%
FGFR3	0	1	0	0	0	0	0	1	13%
HRAS	0	1	0	0	0	0	0	1	50%
EGFR	20		0	2	14	0	0	36	15%
PDGFRA	0	0	0	0	0	0	0	0	0%
AKT1	0	0	0	1	1	0	0	2	67%

Figure 19-Results of molecular analysis according to NGS: Co-mutation

Discussion

Most patients with NSCLC are diagnosed at alate stage, despite recent advances in screening for early lung cancer detection. Fortunately, NGS technology is now widely used to identify cancer gene mutations and provide the molecular basis for appropriate targeted therapy in clinical precision medicine.

In this study based on NGS analysis, we reported a homogeneous dataset of patients affected by advanced NSCLC-A by constructing a complete profile of the genomic alterations of the patients belonging to the S. Orsola Malpighi Hospital.

Basic molecular pre-selection, performed on a small part of the diagnostic biological material, made it possible to optimize the use of the available biological material, avoiding our patients performing further invasive samples and trying not to lose any chance, however much remote access to potentially life-saving targeted drug treatments. Secondly, the initial NGS model offered a less expensive and more reliable diagnostic model than the one used in previous years which was based on a sequential algorithm. Recently Dall' Olio et al demonstrated the feasibility and cost-effectiveness of implementing NGS panels for routine molecular profiling of patients with NSCLC-A [137].

In this study, the genomic signature of lung cancer-related driver genes identified by an NGS-based target sequencing assay showed a higher frequency of rare alterations. Such as a higher prevalence of RET rearrangements than in the literature (3% vs 1%). Alteration of ALK and KRAS also showed a higher prevalence (3% vs 2%) and (33 vs 25%).

In total, 27% of patients had at least one genomic alteration in the druggable genes according to the ESCAT I classification.

Based on these results and thanks to the evaluation with the NGS test developed within the project, many patients in Bologna with rare, advanced lung cancer have had access to targeted oncological therapies for variants not identified during routine tests.

In 410 patients (29%) it was possible to identify rare molecular alterations potentially eligible for advanced therapies with innovative molecularly targeted drugs in the context of clinical trials or extended access programs or "off-label" uses. Of these 20 were enrolled in sponsored clinical trials.

Furthermore, as shown by our next-generation sequencing (NGS) test, a relatively large panel revealed that 31% of cancers carry co-mutations, some of which retain at least prognostic information and a possible mechanism of resistance to chemotherapy drugs. or to molecular targeted agents (MYC, PI3KCA, MAPK, AKT and KIT, HRAS).

PIK3CA mutations frequently coexist with EGFR / KRAS mutations in our study of 11 and 20 patients respectively. PIK3CA mutations may be an adverse prognostic factor in patients with EGFR mutated NSCLC, also Xin Zhou et. al. showed that PI3K inhibition is able to improve the sensitivity of wild-type EGFR NSCLC cell lines to EGFR TKI erlotinib [87,138].

MYC co-mutation in association with ALK-rearranged, or MET-altered patients are involved in primary resistance mechanisms to Crizotinib, cooperating to drive tumorigenesis[139–141]. In these patients, the use of MYC inhibitor together with standard therapies seems promising[142].

Finally, another very promising strategy could be to inhibit CDK4/6 which is also mutated together with MET, KRAS, and EGFR. CDK4/6 inhibitors have been shown to be effective in patients with Osimertinib in EGFR mutated patients [143,144]. Furthermore, in a phase I study of the CDK4/6 inhibitor (Abemaciclib), KRAS mutations appeared to predict disease control in heavily pretreated patients with metastatic NSCLC, with one patient experiencing a partial response[145].

Furthermore, since the list of biomarkers that maintain their predictive value is expanding with the availability of new drugs, for this reason, the need for molecular screening based on multi-gene sequencing will certainly increase in the coming years.

Unfortunately, although we identified 410 potentially eligible patients as they possessed rare mutations, many patients could not be enrolled into clinical trials because they did not meet the harsh criteria for inclusion .

Alternatively, mutations were found prior to the opening of the specific clinical trial, therefore these patients were treated according to clinical practice with the currently available therapies.

Our project has achieved the goal of increasing the standard offer of molecular tests for tumor characterization, carrying out a complete and deep sequencing of tumor DNA using new high-throughput methodologies (Next Generation Sequencing). Consequently, we have increased the opportunity for patients to benefit from current targeted therapies approved by the EMA or currently in clinical trials.

However, many patients cannot access treatment, especially if they are patients with rare mutations for which there is no tailor-made clinical trial.

Atezolizumab in a CoHort of pretreated, advanced, non-small cell lung cancer patients with rare HistologiCal SubtypEs (CHANCE trial)

Introduction

In recent years, ICIs have paved the way for a new era in the treatment of NSCLC, with a significant benefit in terms of overall survival, long-lasting responses, and a better toxicity profile than standard platinum-based chemotherapy.

Notably, nivolumab (anti-PD1 monoclonal antibody) was the first drug in this class to demonstrate prolonged survival when used as second-line treatment, both in lung cancer patients with squamous histology and in patients with non-squamous histology [118].

In patients with chemo-naïve metastatic NSCLC whose, tumors express programmed death-ligand 1 (PD-L1)> 50%, Pembrolizumab, a PD1 monoclonal antibody, has become the new standard of therapy worldwide. More recently, the combination of pembrolizumab or atezolizumab and platinum-based chemotherapy has emerged as an effective first-line treatment regardless of PD-L1 expression in both squamous and non-squamous NSCLC without oncogenic drivers [146]. Moreover, atezolizumab, a monoclonal antibody targeting the PD-1 counterpart, PD-L1, has been approved in NSCLC both alone in PD-L1 high and in combination with carboplatin, paclitaxel, and bevacizumab for non-squamous NSCLC irrespective of PD-L1 expression [125,147].

However, phase 3 studies were focused on common histologies, and little (if any) data is available to decide whether to treat patients with rare NSCLC histologies with these drugs and to inform of their prognosis.

For most rare lung cancers, no standard treatment regimens are available and few case reports of the use of ICI agents have been reported. Due to their low incidence and lack of prospective studies, an effective treatment algorithm remains an unmet need. These considerations highlight the need to evaluate the role of immune checkpoint inhibitors also in the treatment of NSCLC patients with rare histologies. This study aims to explore the antitumor activity and safety profile of atezolizumab in patients with pretreated advanced NSCLC with rare histological subtypes.

Methods

Study design and objectives

This study is an open-label, multicenter, phase II study, designed to evaluate the activity and safety of atezolizumab in a cohort of pretreated, advanced NSCLC patients with rare histological subtypes, which is summarized in Figure 20. The estimated sample size will be approximately 43 subjects, to be enrolled in 11 national centers. The primary objective of this study is to evaluate the activity of atezolizumab in patients with pretreated advanced NSCLC with rare histology subtypes. Secondary objectives are the evaluation of the safety and efficacy of atezolizumab in patients with pretreated advanced NSCLC with rare histology subtypes[148].

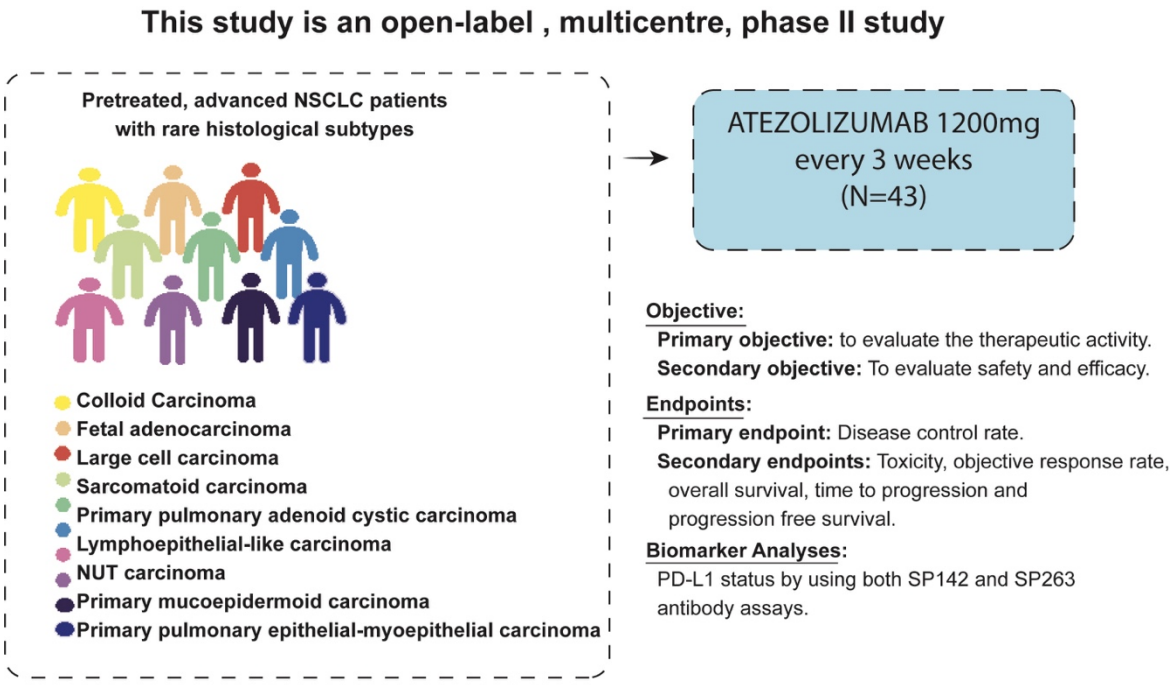


Figure 20-Study design CHANCE Clinical trials[148].

Endpoints

The primary endpoint is an investigator-assessed disease control rate (DCR) according to response evaluation criteria in solid tumors version 1.1 (RECIST v1.1).

Secondary endpoints include toxicity, objective response rate (ORR), overall survival (OS), time to progression (TTP), and progression-free survival (PFS).

Patients

Patients must have a locally advanced, relapsed, or metastatic NSCLC with histologically proven rare subtypes defined according to World Health Organization (WHO) 2015 classification and have experienced disease progression during or after at least one previous standard chemotherapy line. Key Inclusion and Exclusion Criteria are summarized in Figure 21.

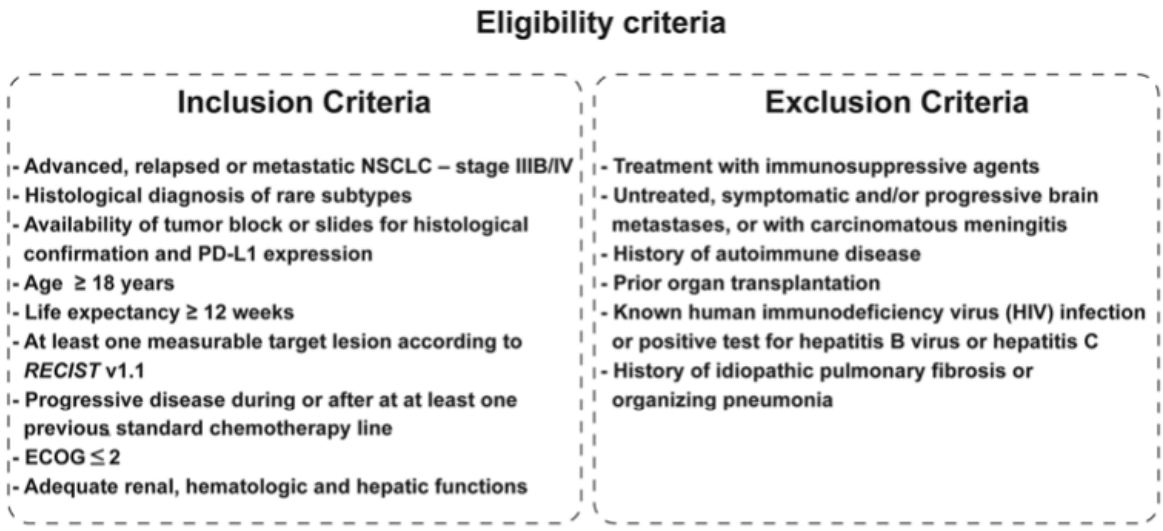
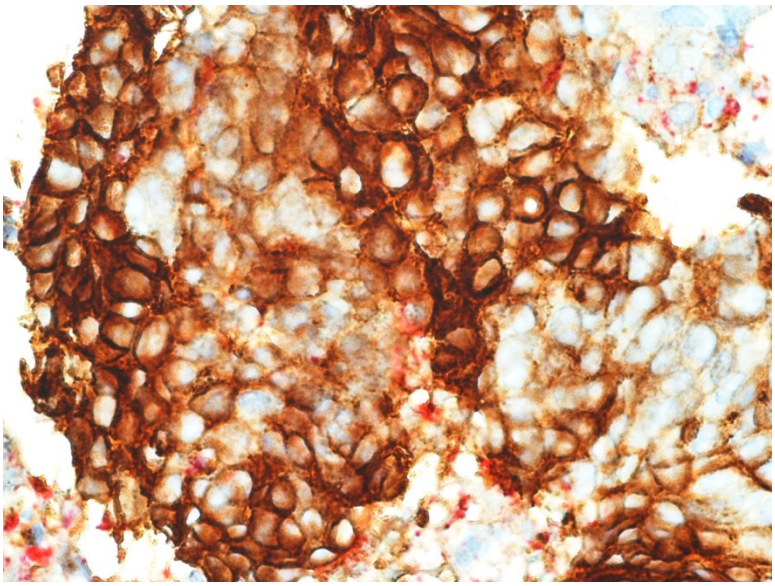


Figure 21-Inclusion and Exclusion Criteria[148]

Molecular analysis

We had planned the evaluation of PD-L1 expression of all tissue samples from enrolled patients using SP-142 and SP-263 assays on ULTRA Automated Immunostainer (Roche). Only samples with a tumor cell count> 100 cells were considered acceptable. Staining assessment was done using the net percentage of positive tumor cells over the entire tumor cell population and the presence and number of positive TILs per high-powered field.

PD- L1 protein expression is determined by using the Tumor Proportion Score (TPS), which is the percentage of viable tumor cells showing partial or complete membrane staining. The specimen should be considered PD-L1 positive if TPS ≥ 50% of the viable tumor cells exhibit membrane staining at any intensity.



$$\text{TPS}(\%) = \frac{\text{No. PD-L1–stained tumor cells}}{\text{Total No. of viable tumor cells}} \times 100$$

Figure 22- Positive PD-L1 membrane staining in NSCLC tumor tissues illustrating and Tumor proportion score (TPS)

Treatment

Treatment consisted of atezolizumab monotherapy. Atezolizumab is a genetically engineered, humanized IgG1 monoclonal antibody to PD-L1 produced in Chinese Hamster Ovary (CHO) cells. Atezolizumab binds to PD-L1 and prevents the interaction between PD-L1 and its receptors PD-1 and B7–1 (or CD80), providing inhibitory signals to T cells. The dose level of atezolizumab proposed to be tested in this study is 1200 mg (equivalent to an average body weight-based dose of 15 mg/kg) administered by IV infusion q3w (±3 days). Treatment will be continued until PD according to RECIST v1.1 criteria, intolerable toxicity, patient refusal, or investigator's decision.

Follow-up and Assessment

Radiological evaluation was performed by computed tomography (CT) every 6 weeks up to 1 year to determine response to treatment using the RECIST v1.1 criteria and every 8 weeks thereafter. At the time of the first tumor evaluation, subjects could continue experimental treatment beyond the initial progressive disease (PD) RECIST v 1.1 in the presence of an established clinical benefit, without rapid disease progression, ≥ 2 - rate increase tumor growth and good tolerance to the study drug. Radiographic reassessment was performed within 6 weeks of the initial investigator-assessed progression to determine if there was a decrease in tumor size or continued PD. For subjects who continued study therapy with atezolizumab beyond progression, further progression is defined as an additional 10% increase in tumor burden volume from the time of initial PD and/or the development of new measurable lesions.

Outcomes

The modified intention to treat the population was analyzed for the primary endpoint and all secondary endpoints. Descriptive tables for tumor response rate and best overall response were produced. We used the exact binomial method to estimate the disease control rate (CR + PR + SD) and its 95% confidence interval. Progression-free survival probabilities and overall survival probabilities will be estimated according to the Kaplan and Meier limit product method. Descriptive toxicity tables have been produced which provide the worst degree of toxicity measured across all cycles according to NCI-CTCAE version 4.03.

Ethical Considerations and Study management

The protocol was written, and the study was conducted according to the Tripartite Harmonized Guidelines for Good Clinical Practice (GCP) of the International Conference on Harmonization (ICH). The study is an independent study sponsored by the Italian Oncology Group of Clinical Research (GOIRC), partially supported by ROCHE with respect to Italian Decree 17 December 2004.

European Clinical Trials Database (EudraCT) number: 2018-002607-34

ClinicalTrials.gov identification number: NCT03976518.

According to the law n. 189 of 08.11.2012, sponsor sent application for authorization to AIFA as the Competent Authority which will carry out the activity within the terms and within the time limits provided for by Legislative Decree 211 (Article 9).

In September 2018 sponsor evaluated the interest of this study of 11 Italian centers, we as a center in Bologna participated in the feasibility expressing our will to participate in the study and to find at least 4 patients to be included in it.

In particular, we were asked for information on the operating unit (staff, structure, patient enrollment process reviews). Bologna site has a clinical trial management office with 6 study coordinators and a research pharmacist who can support clinical research and contacts with promoters also has numerous studies on the lung, but none in conflict with the study proposed by GOIRC . We were therefore selected as the coordinating center of the CHANCE study.

Specifically, we took care of compiling the specific center documentation such as collection of documents of the clinical staff and documentation necessary for the activation of the center.

In meantime, I assisted the GOIRC promoter for the realization of the eCRF in accordance with the protocol.

The validation process was performed according to GAMP 5 and its V-model diagram, starting from the Requirements and Specifications and the corresponding test phase including Functional Tests (OQ).

The Customer must issue a Standard Operating Procedure (SOP) for the internal management of Symphony EDC and perform the necessary tests (Performance

Qualification/User Acceptance) to complete the validation process which began on February 15, 2019 and ended on May 1, 2019 with the final release of e-CRF CHANCE GOIRC-02-2018 and user manual for all centers.

The eCRF consists of several pages to be filled in online during the visits, information was collected on informed consent, demographics, vital signs, laboratory parameters including PD-L1 status, study drug infusion anamnesis, tumor history, tumor assessment concomitant drugs and events adverse. a specific page was also created for the interruption visit from the study in which to insert the reason for the interruption and a page of FU where the subsequent therapies and the survival data.

Table 4 shows the 13 Italian centers activated by GOIRC.

N° Centro	City	PI	Data CE Approval	Data SIV
1	Azienda Ospedaliero- Universitaria Sant'Orsola Malpighi di Bologna UOC di Oncologia Medica- Bologna	Ardizzoni	20/02/19	07/05/2019
2	AORN Ospedali dei Colli – V. Monaldi U.O.C Oncologia Medica- Napoli	Rocco	07/05/2019	22/10/2019
3	Azienda Ospedaliero-Universitaria di Parma UOC Oncologia Medica- Parma	Tiseo	18/03/19	10/07/2019
4	Azienda Ospedaliera Universitaria San Martino di Genova U.O Oncologia Medica- Genova	Genova	NA	NA
5	Istituto Oncologico Veneto IOV-IRCCS- U.O. Oncologia 2- Padova	Pasello	15/04/19	15/07/2019
6	IFO- Istituto regina Elena- C.S. Oncologia Medica- ROMA	Vari	29/01/19	20/06/2019
7	AOU Pisana- U.O. Pneumologia- Pisa	Chella	28/03/19	03/07/2019
8	Fondazione IRCCS Istituto nazionale Tumori-Milano S.S. Oncologia medica Toraco-polmonare– Milano	Garassino	26/02/19	02/08/2019
9	Azienda Sanitaria Universitaria integrata di Udine U.O Oncologia- Udine	Macerelli	02/04/19	18/07/2019
10	AOU San Luigi Gonzaga di Orbassano-Torino U.O Oncologia polmonare	Novello	16/01/19	29/10/2019
11	AO PERUGIA SC Oncologia Medica – Perugia	Roila	13/12/18	18/07/2019
12	IRCCS-Cà Granda Ospedale Maggiore-Milano UO Oncologia medica	Grossi	16/07/19	16/12/2019
13	A.O. S. Croce e Carle Ospedale Carle Oncologia Medica - Cuneo	Colantonio	20/06/19	19/11/2019

Table 5 – Site on CHANCE Clinical trials[149]

Procedure for starting CHANCE Clinical trials of the coordinating center in Bologna is summarize on figure 23.

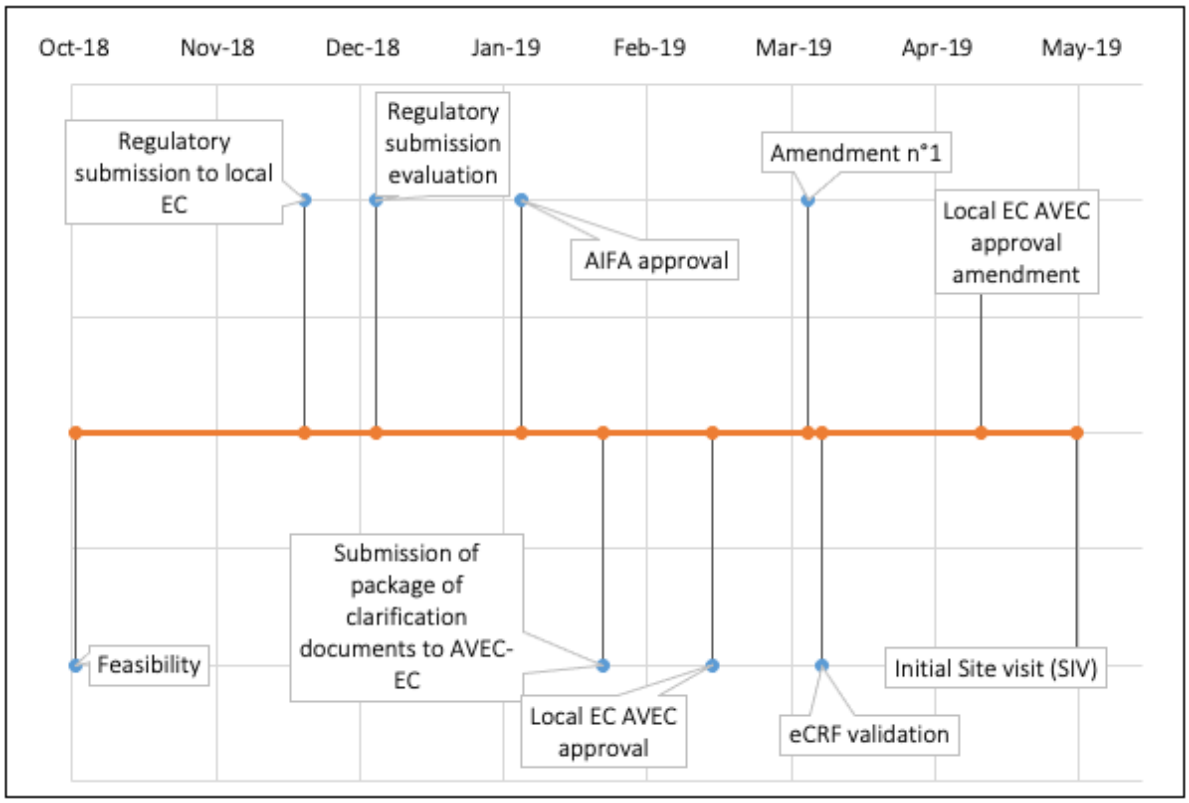


Figure 23- Regulatory process for starting CHANCE clinical trials.

Study was approved by the Italian agency AIFA on January 2019.

On 12 March 2019, a substantial change was necessary regarding the update to the version of the atezolizumab Investigator V.14 brochure and an important Security update of 24 January 2019. These changes made it necessary to update the protocol and at the same time GOIRC have also increased the number of centers from 11 to 13. This is the Dear Investigator Letter dated 24/01/2019 sent by Roche Spa regarding an important safety update.

As part of the analyzes conducted with atezolizumab, some cases of immune-related myositis were observed. Four of these events had a fatal outcome. The incidence in the atezolizumab monotherapy clinical program was <0.1%. The amendment was approved by AIFA on 12 April 2019 and the single favorable opinion of the coordination center was issued on 17 April 2019. We enrolled the first patient directly with the amended protocol and the new consents reporting this important adverse event.

After some clarifications that emerged on December 2018, ethics committee of the AVEC coordinating center approval this study on February 2019 (CE 748/2018/Farm/AOUBo). Site of Bologna had its initial site visit (SIV) on May 2019.

The first patient was enrolled on 07 May 2019 in coordinating center of Bologna. GOIRC enrolled 35 patients. In table 5 you can see all CHANCE enrollment trend and enrollment on bologna site, unfortunately pandemic situation of COVID 19 which arose in February 2020 has greatly reduced the inclusion of patients in this study. Indeed, as it can be seen from table 5 in the lockdown period (March 2020 - May 2020) or in the period in which major infections COVID-19 occurred (October 2020 - May 2021), enrollment remained unchanged due to logistical difficulties for patients with rare cancers residing away from the nursing hospital has created a serious obstacle to the inclusion of new patients in clinical trials.

Furthermore, an AIFA communication of 7 April 2020 provides information on the management of clinical trials and on substantial changes in Italy following the exceptional restrictive measures introduced by the Italian government in the context of the fight against the COVID-19 pandemic. Regarding enrollment, the communication stressed that the inclusion and enrollment of new patients in clinical trials should be avoided as much as possible except in cases where participation in the study is of fundamental necessity, as in the absence of a valid therapeutic alternative [150].

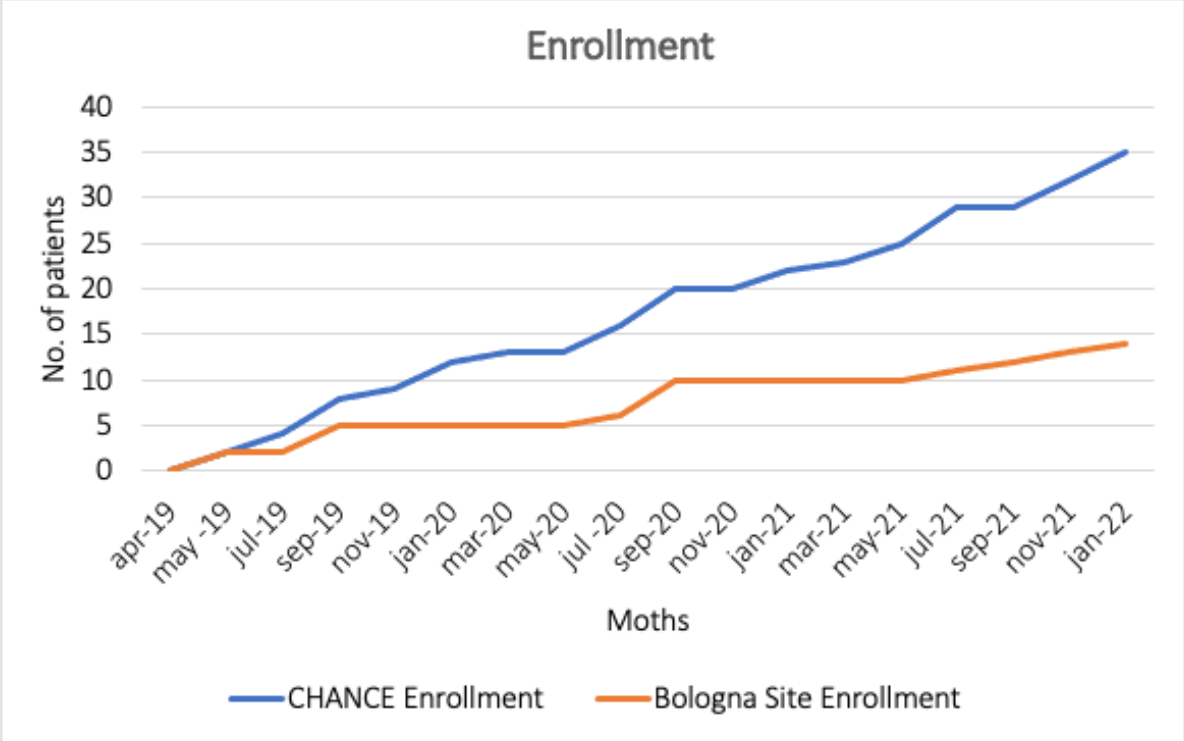


Table 6- Enrollment of study CHANCE

Results

Between May 7, 2019, and December 31, 2021, Policlinic S.Orsola Malpighi have been enrolled 14 patients into the trial. Data are presented up to January 11, 2022.

This patients with rare lung histology were evaluated in Bologna, predominantly men (69%) with a mean age of 71 years (48-80), most of whom were former smokers (64%) or never smokers (21%). Finally, all patients had an ECOG performance status score of 0 or 1 and had stage IV cancer. The characteristics of the population in question are summarized in table 6.

Characteristic- Bologna		No.	%
Age, Median (range)		71 (48-80)	
Gender:			
	Male	9	64%
	Female	5	36%
Smoking Status			
	Never smoked	3	21%
	Former smoker	9	64%
	Current smoker	2	14%
ECOG performance-status score			
	0	7	50%
	1	7	50%
	2	0	0%

Table 7 Characteristics of coordinator center of Bologna

Almost one third enrolled patients showed a type of large cell neuroendocrine carcinoma LCNEC histology, in table 7 you can see the types of histologies found in the patients enrolled in the study.

Histological type			
	adenoid-cystic carcinoma of the trachea	1	7%
	Primary pulmonary adenoid cystic carcinoma	1	7%
	adenoid cystic carcinoma of trachea	1	7%
	large cell neuroendocrine carcinoma (LCNEC)	5	36%
	Sarcomatoid carcinoma	2	14%

Table 8- Histological type

Atezolizumab immunotherapy was given as second-line treatment in 71% of patients and as third-line or higher treatment in 14% of patients, while radiotherapy was done only in 43% of patients. The most frequently type of prior systemic therapy administered was carboplatin plus paclitaxel or carboplatin plus etoposide. All patients underwent a CT scan before the start of therapy to assess the radiological activity of the disease. The main sites of disease are lung, lymph nodes , liver, brain, pancreas, bone , pleura and other sites (Table 9).

No. of prior systemic regimens			
	1	10	71%
	2	2	14%
	3	2	14%
Radiotherapy			
	YES	6	43%
	NO	8	57%
Anatomical localization of RECIST 1.1			
	Lung	7	
	Liver	8	
	lymph nodes	5	
	Brain	1	
	Pancreas	1	
	Bone	3	
	Pleura	2	
	Other	2	

Table 9- Prior therapy and Anatomical localization of tumor

14 patients treated were assessable for toxicity. Treatment-related adverse events were predominantly grade 1 or grade 2 (table 10). The most common grade 3 treatment-related adverse events were GGT increase (14%), fatigue (7%), anaemia (7%), diarrhoea (7%), increased alanine aminotransferase (7%) and hyperglycaemia (21%). Only one patient had Grade 4 GGT elevation for which he had temporary discontinuation of therapy.

Adverse Events	Common Terminology Criteria for Adverse Events (CTCAE)				
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
GGT increased	4 (28%)	0	2 (14%)	1 (7%)	0
Enema	4 (28%)	0	0	0	0
Abdominal Pain	1 (7%)	0	0	0	0
fever	2 (14%)	0	0	0	0
Fatigue	3 (21%)	3 (21%)	1 (7%)	0	0
Anaemia	0	1 (7%)	1 (7%)	0	0
diarrhoea	3 (21%)		1 (7%)	0	0
Loss of appetite	4 (28%)	3	0	0	0
Dyspnoea	4 (28%)	2 (14%)	0	0	0

Depression	1 (7%)	0	0	0	0
dysgeusia	1 (7%)	1 (7%)	0	0	0
Musculoskeletal Pain	4 (28%)	3 (21%)	0	0	0
dizziness	0	1 (7%)	0	0	0
Aspartate aminotransferase increased	2 (14%)	1 (7%)	0	0	0
Alanine aminotransferase increased	2 (14%)	0	1 (7%)	0	0
xerostomia	1 (7%)	0	0	0	0
Blood bilirubin increased	1 (7%)	1 (7%)	0	0	0
FT4 increased	1 (7%)	1 (7%)	0	0	0
TSH increased	1 (7%)	2 (14%)	0	0	0
hyperglycaemia	0	1 (7%)	3	0	0
creatine increased	1 (7%)	1 (7%)	0	0	0
cough	1 (7%)	0	0	0	0
dysphagia	3 (21%)	1 (7%)	0	0	0
right hypochondrium pain	1 (7%)	0	0	0	0
hypercalcemia	0	1 (7%)	0	0	0
Epistaxis	1 (7%)	0	0	0	0
Hypotension	1 (7%)	1 (7%)	0	0	0
Periodontal disease	0	1 (7%)	0	0	0
suspected pneumonia	0	1 (7%)	0	0	0
Superficial venous circles	1 (7%)	0	0	0	0
Scleral hyperaemia	1 (7%)	0	0	0	0
lumbar Rash macular	1 (7%)	0	0	0	0
pharyngal pain	1 (7%)	0	0	0	0
Faecal incontinence	1 (7%)	0	0	0	0
apnoea	0	1 (7%)	0	0	0
Paresthesia	2 (14%)	0	0	0	0
Alopecia	1 (7%)	1 (7%)	0	0	0
hypertension	1 (7%)	0	0	0	0
Alkaline phosphatase increased	0	1 (7%)	0	0	0
Right hand Redness	1 (7%)	0	0	0	0
Right hand Pruritus	1 (7%)	0	0	0	0

Table 10- Treatment-related toxicities of atezolizumab

Discussion

These neoplasms are characterized by an intrinsic difficulty in identifying which are the most appropriate care modalities in these specific clinical conditions.

The limited number of cases found in the population, in fact, requires careful multidisciplinary evaluation.

The management of rare lung cancers still lacks guidelines that illustrate global recommendations for clinical behavior to be followed, therefore the therapeutic choice is decided by an expert based on the specific situation and the basis of the therapies available.

Indeed, although the role of ICI immunotherapy is now well established in the treatment of NSCLC, there is very little data on the treatment in NSCLC of rare histology and it is not known whether these treatments may be an option for these patients.

Only a few studies have reported a clinical benefit of ICIs in patients with sarcomatoid lung cancer and lymphoepithelioma[151–153].

The phase II study (DART SWOG1609) is investigating the efficacy of anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) combined therapy in rare cancers, including those of pulmonary origin[154].

In conclusion, rare lung tumors acquire a non-negligible portion of the entire oncological pathology.

The need therefore remains to implement specific clinical protocols dedicated to rare situations, which will allow us to study the efficacy of these indicated treatments with the hope of expanding the therapeutic range in the future.

Currently, CHANCE study has enrolled 35 patients throughout Italy and in the Bologna site it has enrolled 14 patients, giving these people a therapeutic option that was previously precluded. As enrollment of patients in the CHANCE study is still ongoing, no definitive conclusions on the effectiveness of ICIs on rare lung cancer hystologies can be drawn.

Phase II Study With Cabozantinib in Patients With RET Positive NSCLC (CRETA)

Introduction

Targeted therapy plays a key role in mutations in cell cycle surveillance, survival, and inhibition of apoptosis genes. More recently, some RET fusion genes (KIF5B-RET, CCDC6-RET, NCOA4-RET) have been identified as oncogenic drivers in 1-2% of NSCLC, while RET rearrangement was detected in 2.2% of patients with triple wild-type lung adenocarcinoma for KRAS, EGFR and ALK [155].

RET-rearranged NSCLC tumors have peculiar clinical features such as younger age, predominantly female gender, low exposure to cigarette smoke, rapid metastatic spread (including CNS), almost exclusively adenocarcinomatous histology. From a therapeutic point of view, RET-rearranged tumors seem to respond to platinum-based chemotherapy (especially the combination of platinum and pemetrexed) while they are relatively resistant to immunotherapy. Clinically, the benefit of the more recently developed RET inhibitors, selpercatinib and pralsetinib, has been demonstrated in terms of response and median progression-free survival [156157]. Cabozantinib, a multitarget tyrosine kinase inhibitor, was studied in a single small phase II study that enrolled 26 patients, documenting a response rate of 28% and a PFS of 5.5 months[158]. The CRETA study was designed to evaluate the activity and safety of cabozantinib in patients with RET-rearranged NSCLC pre-treated with conventional chemotherapy.

Methods

Study design and objectives

This study is an open-label, multicentre, phase II study, designated for evaluating the activity and safety of cabozantinib in a cohort of pretreated, advanced NSCLC patients with advanced RET-rearranged non-small cell lung cancer. It is planned to recruit approximately 25 subjects at up to 11 national centers.

- The primary objective of this study is to evaluate activity of cabozantinib in patients with advanced RET-rearranged non-small cell lung cancer (NSCLC).
- Secondary objectives are evaluation of safety and efficacy of cabozantinib in pretreated patients with advanced RET-rearranged non-small cell lung cancer (NSCLC)
- Exploratory objectives of this study is to detect potential mechanisms of acquired resistance to RET inhibition.

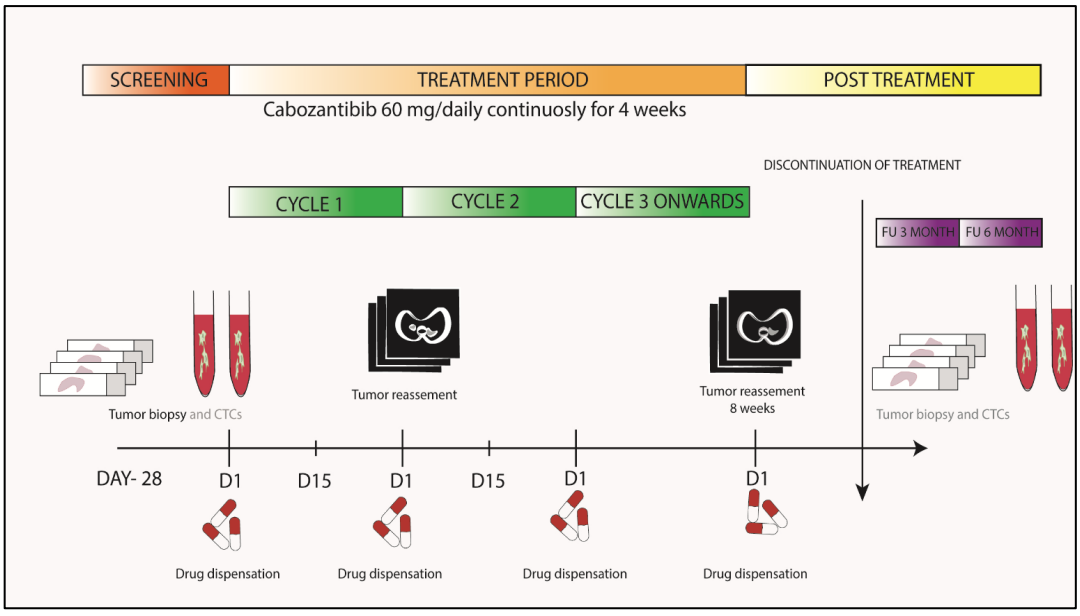


Figure 24- Study design of CRETA clinical trials

Endpoints

Primary Endpoint

The primary end-point is objective tumor response. Tumor responses will be evaluated according to standard RECIST 1.1 criteria. Data will be reported as percentage of complete responses (CRs), partial responses (PRs), stable disease (SD) and progressive disease (PD), as assessed by central independent imaging review. Exact binomial method will be used to estimate the response rate (CR+PR) and its 95% confidence interval. Patients with no tumor assessment after baseline will be classified as non-responders.

Secondary Endpoints

The secondary endpoints are:

- Toxicity: the assessment of safety will be based mainly on the frequency of adverse events; toxicity will be measured according to NCI Common Toxicity Criteria Adverse Event (CTCAE), version 4.03.
- Progression-Free Survival (PFS) will be calculated from the first treatment intake to the date of progressive disease, or death.
- Overall survival (OS) will be calculated from the first treatment intake to death from any cause.
- Duration of response (DOR) will be calculated from the first treatment intake to disease progression or death date.
- Disease Control Rate (DCR) will be measured as the sum of complete and partial responses + stable disease.

Patients

Inclusion criteria

Study subjects must meet all of the following criteria to be considered for inclusion:

1. Locally advanced, relapsed or metastatic non-small cell lung cancer – stage IIIB/IV according to 7th International Association for the Study of Lung Cancer (IASLC) classification.
2. Pathologically (histology or cytology) confirmed diagnosis of non-small cell lung carcinoma
3. RET gene rearrangement determined by local laboratory analysis with an approved standard method (FISH or Next Generation Sequencing Panel). An archival tumor sample must be available for central laboratory confirmation.
4. Have progressed after or during at least one standard anticancer treatment
5. Have measurable disease as per Response Evaluation Criteria in Solid Tumors, version 1.1 (RECIST 1.1); clear radiological evidence of disease progression after first-line therapy must be documented; no previous radiotherapy on the only site of measurable or evaluable disease, unless that site had subsequent evidence of progression
6. Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) of 0-1
7. No radiologic or clinical evidence of acute or chronic pancreatitis
8. Females must be postmenopausal; furthermore, patients must agree to adopt 2 effective methods of contraception. Males even if surgically sterilized agree to practice effective barrier contraception during the entire study treatment period and through 4 months after the last dose of study drug.

9. Subjects must have adequate organ function including the following:

- Absolute neutrophil count > 1.5 x 10^9/L
- Platelet count > 100 x 10^9/L
- Haemoglobin > 90 g/L
- ALT < 2.5 times the upper limit of normal (ULN)
- AST < 2.5 times ULN
- Total bilirubin <1.5 times ULN
- Creatinine <1.5 times ULN concurrent with creatinine clearance > 50 ml/min (measured or calculated by Cockcroft and Gault equation, confirmation of creatinine clearance is only required when creatinine is > 1.5 times ULN)
- Lipase < 2.0 times the upper limit of normal (ULN)

Exclusion criteria

Patients meeting any of the following exclusion criteria are not eligible for the study:

1. Radiation therapy for bone metastasis within 2 weeks, any other external radiation therapy within 4 weeks before randomization. Systemic treatment with radionuclides within 6 weeks before randomization. Subjects with clinically relevant ongoing complications from prior radiation therapy are not eligible.
2. Previous treatment with Cabozantinib
3. Gastrointestinal disorders.
4. Current use of aspirin, clopidogrel, ticlopidine.

5. Patients with tumors invading major pulmonary vessels and/or with cavitating pulmonary lesions.
6. Major surgery (eg, GI surgery, removal or biopsy of brain metastasis) within the last four weeks. Complete wound healing from major surgery must have occurred 1 month before randomization and from minor surgery (eg, simple excision, tooth extraction) at least 10 days before randomization. Subjects with clinically relevant ongoing complications from prior surgery are not eligible.
7. Subjects with clinical or radiological signs of pulmonary hemorrhage within 3 months before the first dose of study treatment.
8. Symptomatic CNS or leptomeningeal lesions, not previously treated with radiotherapy.
9. History of congenital platelet function defect (e.g., Bernard-Soulier syndrome, Chediak-Higashi syndrome, Glanzmann thrombasthenia, storage pool defect).
10. Patient unable to swallow tablets
11. Corrected QT interval greater than 500 ms (Fridericia formula)
12. Clinically significant, uncontrolled heart diseases:
13. Unstable angina within 6 months prior to screening
14. Myocardial infarction within 6 months prior to screening
15. History of documented congestive heart failure (New York Heart Association functional classification III-IV)
16. Uncontrolled hypertension defined by a Systolic Blood Pressure (SBP) ≥ 160 mm Hg and/or Diastolic Blood Pressure (DBP) ≥ 100 mm Hg, with or without

antihypertensive medication. Initiation or adjustment of antihypertensive medication(s) is allowed prior to screening

17. Ventricular arrhythmias
18. Supraventricular and nodal arrhythmias not controlled with medication
19. Congenital history of QT syndrome
20. History of artery dissections or aneurysms.
21. Diagnosed with or treated for another malignancy within 3 years before the first dose of study drug, or previously diagnosed with another malignancy and have any evidence of residual disease. Patients with non-melanoma skin cancer or carcinoma in situ of any type may be enrolled in the study if they have undergone complete resection and no evidence of active disease is present.
22. Any type of systemic anticancer agent (including investigational) within 3 weeks of first dose of study treatment, or within 5 half-lives of the agent whichever is shorter (subjects on LHRH or GnRH agonists may be maintained on these agents).
23. Any serious and/or unstable pre-existing medical, psychiatric, or other conditions that could interfere with the subject's safety, provision of informed consent, or compliance to study procedures.
24. Rare hereditary problems of galactose intolerance, the Lapp lactase deficiency or glucose-galactose malabsorption.

Molecular analysis

The rearrangement of the RET genus determined by an approved standard evaluation method (FISH or Next Generation Sequencing Panel).

We used a two-color break-apart fluorescence in situ hybridization assay using Vysis 10q11 RET Break-Apart FISH Probe Kit (Abbott Diagnostics Inc.).

Alternatively, we used Oncomine Focus Assay Gene Panel - Thermo Fisher Scientific, Carlsbad, CA (RUO Kit) to perform next-generation sequencing of tumor DNA.

The standard operating protocol of the FISH technique includes several phases;

It starts with the availability of tissues (surgical samples, biopsies, cell blocks, and clots) fixed in formalin and embedded in FFPE paraffin. From this block 4 um thick sections will be obtained on loaded slides (at least 5 slides) containing a minimum of 50 infiltrating tumor cells and not older than 6 years.

Then we move on to dewaxing, rehydration, thermal and enzymatic pre-treatment, the probes are incubated strictly in the dark. The slides are then placed in ThermoBrite where the denaturation and hybridization of the probes take place. The following day, after washing to eliminate the non-hybridized probes, the DAPI counterstain (4 ', 6-diamidine-2-phenylindole) is added; the slides are finally sealed and stored in the dark at 4 ° C. FFPE slides will be analyzed using a Nikon Eclipse 80i microscope equipped with a triple pass filter (DAPI / Green / Red). The FISH results will be evaluated by two independent expert operators. The split RET signal will be considered separated by at least two length diameters. The case will be considered positive for RET rearrangement when at least 15% of the cells will contain RET division signals.

Treatment

Eligible patients have been registered to receive cabozantinib orally which will be administered at an initial dose of 60mg daily in tablet form.

The drug is taken continuously for 28 days or 4 weeks. This is defined as one treatment cycle. Cabozantinib should be taken in fasting condition with no food for at least 2 hours before and 1 hour after taking the tablets. Each patient is only allowed two dose reductions (40mg or 20 mg).

Treatment is continued until progression of the disease, significant toxicity, subject withdrawal, or subject removal. The response is monitored by serial imaging and

measured by RECIST v1.1 and will be based on central imaging assessment and review by Independent Review.

Follow-up and Assessment

Radiological evaluation was performed by computed tomography (CT) every 6 weeks up to 1 year to determine response to treatment using the RECIST v1.1 criteria and every 8 weeks thereafter. At the time of the first tumor evaluation, subjects could continue experimental treatment beyond the initial progressive disease (PD) RECIST v 1.1.

Outcomes

The primary objective of the study is to demonstrate the antitumor activity of cabozantinib in pretreated patients with advanced RET-rearranged NSCLC, as measured by overall response rate (ORR) (CR+PR) according to RECIST v1.1 criteria.
Secondary objectives include safety, Progression-Free Survival (PFS), overall survival (OS), Duration of Response (DOR) and Disease Control Rate (DCR).

- *Safety evaluation: the assessment of safety will be based mainly on the frequency of adverse events. Adverse events will be summarized by presenting the number and percentage of patients having any adverse event, having an adverse event in each body system and having each individual adverse event.*
- PFS will be calculated from the first treatment intake to the disease progression or death date.
- *OS will be calculated from the first treatment intake to the date of death from any cause.*
- DOR will be calculated from the first treatment intake to the disease progression or death date.
- DCR will be measured as the sum of complete and partial responses + stable disease.

Ethical Considerations and Study management

The protocol was written and the study was conducted according to the Tripartite Harmonized Guidelines for Good Clinical Practice (GCP) of the International Conference on Harmonization (ICH). The study is an independent study sponsored by Alma Mater Studiorum - University of Bologna, which received an unlimited grant from Ipsen. The clinical trial was registered in OsSC and the European Clinical Trials Database (EudraCT): 2018-002948-88 under number and with ClinicalTrials.gov identification number: NCT04131543.

On 26 June 2018 the University Of Bologna went along with the proposal as regards finance by IPSEN S.p.A. In September 2018 we evaluated the interest of 11 Italian centers in joining the study in question. In the table you can find the indicative number of patients eligible for this study centers.

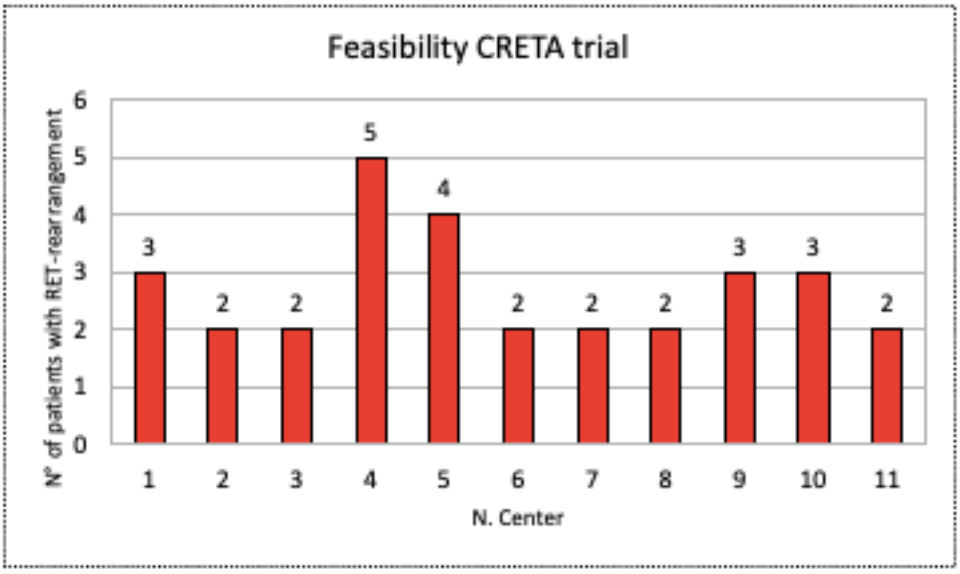


Table 11- Feasibility CRETA trials

The insurance for the CRETA study was finalized with the help of the DIMES Office for the Management of Insurance Contracts on 06 November 2019.

Informed consent and all patient documentation were obtained using the model provided by the committee and the information and privacy consent form formed by DIMES - Privacy Office. The paper documentation was mailed to all satellite centers and their ethics committees.

The project was presented through OsSC (Observatory on clinical studies) by the referent of the Alma Mater Studiorum University of Bologna for clinical trials on November 28, 2018 and

AIFA approval clinical study on January 29, 2019.

The ethics committee of the coordinating center reviewed the documentation on 29 January 2018. After some clarifications that emerged on 14 December 2018, on 20 February 2019 ethics committee of the coordination center AVEC issued a positive opinion.

To guarantee all the study procedures and databases, we have created tenders for purchase in collaboration with DIMES: purchasing office for management and drug, monitoring and pharmacovigilance services and eCRF.

Furthermore, before the opening of the centers, the contracts with DIMES Servizi alla ricerca were finalized.

The coordinating center was opened (SIV) on 7 August 2019 and the first patient was enrolled on 9 August 2019. Table 12 summarizes the centers and the opening visits of the centers with the number of patients enrolled.

N.	City	PI	Date of Approval	SIV	No. Subject
1	BOLOGNA	Ardizzoni	20/02/19	07/08/2019	3
2	PARMA	Tiseo	21/05/19	03/07/2020	0
3	MILANO	Garassino	17/12/19	20/11/20	0
4	PADOVA	Pasello	16/12/19	28/07/2020	0
5	UDINE	Rossetto	22/10/19	06/11/20	1
6	PISA	Chella	NA	NA	NA
7	ROMA	Vari	NA	04/02/20	1
8	NAPOLI	Rocco	18/09/19	09/06/20	0
9	CATANIA	Soto Parra	15/04/19	NA	NA
10	PERUGIA	Roila	13/02/18	NA	NA
11	GENOVA	Genova	18/05/20	NA	NA

Table 12-Approval process of the CRETA study and patient enrollment status.

Subsequently, on June 14, 2019, a non-substantial amendment was necessary to change two Principal Investigators from Genova and Rome, replaced respectively by Dr. Genova and Dr. Vari. CRETA study was concluded in 13 may 2021 and did not reach the patient target number.

Results

Five patients were enrolled between August 2019 and February 2021.

They were predominantly female, with PS 1 and with an age varying between 42 and 72 years, in stage IIIB-IV of the disease (including 2 cases with brain metastases) . Before Cabozantinib treatment, all patients received 2-3 previous lines of chemotherapy regimen. All enrollment patients had a RET rearrangend KF5B(15)-RET(12).

The patients' clinical characteristics are provided in Table 12.

Characteristic		No.	%
Age,Median (range)		50 (42-72)	
Gender:			
	Male	1	20%
	Female	4	80%
Smoking Status			
	Never smoked	1	20%
	Former smoker	3	60%
	Current smoker	1	20%
ECOG performance-status score			
	0	0	0%
	1	4	80%
	2	0	0%
Stage			
	IIB	1	20%
	IV	4	80%
No. of prior systemic regimens			

	1	2	40%
	2	1	20%
	3	2	40%
Radiotherapy			
	YES	2	40%
	NO	3	60%
Anatomical localization of RECIST 1.1			
	Lung	5	
	Liver	2	
	lymph nodes	4	
	Brain	2	
	Bone	2	
	Pleura	1	
	Other	1	

Table 13-Clinical characteristics of subject enrollemed in CRETA trials

Treatment was overall well tolerated although some temporary suspensions and dose reductions were required in some patients due to toxicity.

Treatment-related toxicities of cabozantinib arising during experimental treatment are summarized in Table 16.

Treatment-related adverse events were predominantly grade 1 or grade 2 (table 16). The most common grade 3 treatment-related adverse events were amilase increase (40%), lipase increase (20%), TSH increase (20%). There were no grade 4 adverse events.

Adverse Events	Common Terminology Criteria for Adverse Events (CTCAE)				
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
hypertension	1	2			
asthenia	1				
neutropenia		1			
dysphonia	2				
gastroesophageal reflux	1				
amilase increase	2		2		
lipase increase	1	2	1		
fever	1				
TSH increase	2		1		
leukopenia		1			

diarrhea	3	1			
alopecia	2				
hair depigmentation	2				
tachycardia	1				
dryness of mucosa	1				
creatinina increase	1				
dysgeusia	1				
stomatitis	1				
HFS	1				
hypertransaminasenia	2	2			
Left back pain	1				
onicopatia	1				
dry cough	1				
dyspnea	1				
Paresthesias	1				
weight loss	1				
thrombocytopenia	1				
increased bilirubin	1	1			
hypothyroidism	1				
lack of appetite	1				
hemicostatic pain	1				

Table 16- Treatment-related toxicities of cabozantinib

Discussion

The limited number of cases does not allow definitive conclusions to be drawn on the objectives set by the CRETA study. Although on a very limited series, cabozantinib treatment still appears tolerable and has some antitumor activity.

The goal of the study was to reach a recruitment of 25 patients by 11 active clinical centers over a period of 24 months. Unfortunately, only 5 patients were enrolled in the first 22 months of opening the centers, representing about 20% of the expected recruitment.

The low recruitment is linked to the fact that the therapeutic scenario of this type of disease has drastically changed in the last two years after the opening of the CRETA study. In 2019, the results of phase I/II studies conducted with two new selective inhibitors of the RET gene, selpercatinib and pralsetinib, were disclosed, indicating an efficacy superior to cabozantinib [133,156,159].

These drugs were approved in 2020 by the Food and Drug Administration for the treatment of patients with advanced or metastatic NSCLC positive for RET fusion. These selective RET inhibitors are now available through off-label uses or clinical studies conducted in Italy and Europe and represent a preferable clinical option over the CRETA study, making the clinical question underlying this project obsolete. In this scenario, the main criticality encountered is the time factor, in fact research runs fast and bureaucracy often prevents us from keeping up with clinical research in a cosmopolitan world. In our project, observing table 11, we realize how much time has passed from the idea of the project (November 2018) to the opening of the last clinical center (November 2020).

About 2 years to obtain approval from regulatory bodies, finalization of contracts and opening of 7 out of 11 total centers planned.

In research competition, the Italian bureaucracy is still too slow in an ever-winning European context.

CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

Lung cancer is an extremely heterogeneous disease both in terms of its molecularaspect and histological carachteristics. NGS methods have proved to be applicable in the clinical routine of a university hospital like ours.

In 410/1408 patients (29%) the NGS analysis showed the presence of an actionable rare molecular alteration (RET, NTRK, FGFR1, MET exon14 skipping, BRAF, ALK, EGFR exon 20) possibly treatable with new molecular targeted agents

Uncommon variants were found in 36/1408 patients (2%), some of which were associated with possible resistance to target drugs or chemotherapy.

Thanks to these results, 22 patients with A-NSCLC had access to experimental therapeutic protocols. This first part of the project demonstrated how accurate screening is necessary to match the right therapy to the patient's molecular profile.

The classification of the World health organization (WHO classification) subcategories the different types of lung tumors in relation to their different origin (epithelial, mesenchymal) and degrees (malignant and benign). Some of these, such as lung adenocarcinoma (AC) and squamous cell lung cancer (SCC), are very frequent while other histologies are defined as rare (<1%). Currently, very little is known about these histologies and their therapeutic efficacy. In the second part of my project, I worked on creating a study that would allow 14 of these patients to be treated with an ICIs to evaluate the therapeutic efficacy and expand the scientific knowledge of the treatment of these subpopulations.

Finally, in the CRETA study, we included 5 patients who were treated with a nonselective molecular target drug.

In this last part of my doctoral project, a major problem of the Italian reality emerged, the slowness of the bureaucracy compared to the speed of discovery at a scientific level, making collective efforts to give patients the best therapy on the market useless.

In conclusion, this project made it possible to study the biology of rare lung tumors in more detail, identifying suitable subpopulations for different therapeutic strategies.

In the future it will be increasingly necessary to use this approach in order to propose to each patient the best feasible therapeutic strategy.

LIST OF SCIENTIFIC PAPERS

Emerging Novel Therapeutic Approaches for Treatment of Advanced Cutaneous Melanoma.

F. Comito, R. Pagani, G. Grilli, F. Sperandi, A. Ardizzoni and B. Melotti Cancer 2022; doi.org/10.3390/cancers14020271

Monitoring tumor growth rate to predict immune checkpoint inhibitors' treatment outcome in advanced NSCLC.

F.G. Dall'Olio, C. Parisi, L. Marcolin, S. Brocchi, C. Caramella, N. Conci, G. Carpani, F. Gelsomino, S. Ardizzoni, P.V. Marchese, A. Paccapelo, G. Grilli, R. Golfieri, B. Besse, A. Ardizzoni. Ther Adv Med Oncol 2022 (accepted, in press).

Comparison of Sequential Testing and Next Generation Sequencing in advanced Lung Adenocarcinoma patients - A single centre experience.

F.G. Dall'Olio , N. Conci , G. Rossi , M. Fiorentino , A. De Giglio , **G. Grilli** , A. Altimari , E. Gruppioni , D.M. Filippini , A. Di Federico , A.Ardizzoni *Lung Cancer*. 2020;149:5-9

Atezolizumab in a CoHort of pretreated, advanced, non-small cell lung cancer patients with rare HistologiCal SubtypEs (CHANCE trial).

Gelsomino F, Lamberti G, Tiseo M, Rocco D, Pasello G, Cecere FL, Chella A, Grilli G, Mandruzzato M, Tognetto M, Garassino MC, Macerelli M, Novello S, Roila F, Colantonio

I, Grossi F, Fiorentino M, Ardizzoni A.Ther Adv Med Oncol. 2020 Jul 20;12:1758835920915983. doi: 10.1177/1758835920915983. eCollection 2020.

Drug-induced colitis on BRAF and MEK inhibitors for BRAF V600E-mutated non-small cell lung cancer: a case report.

Gelsomino F, Di Federico A, Tardio ML, Grilli G, D'Errico A, Ardizzoni A, Salvagni S. Invest New Drugs. 2021 Aug 26.

PD-L1 Expression in Circulating Tumor Cells as a Promising Prognostic Biomarker in Advanced Non-small-cell Lung Cancer Treated with Immune Checkpoint Inhibitors.

Dall'Olio FG, Gelsomino F, Conci N, Marcolin L, De Giglio A, Grilli G, Sperandi F, Fontana F, Terracciano M, Fragomeno B, Tober N, Manferrari G, Brocchi S, Golfieri R, Fiorentino M, Ardizzoni A. Clin Lung Cancer.21 Mar16

Comparative effectiveness of first-line immune checkpoint inhibitors plus tyrosine kinase inhibitors according to IMDC risk groups in metastatic renal cell carcinoma: a meta-analysis.

Rizzo A, Mollica V, Santoni M, Rosellini M, Marchetti A, Ricci AD, Grilli G, Greco A, Montironi R, Ardizzoni A, Massari F. Immunotherapy. 2021 Jun;13(9):783-793.

Roles of IL-1 in Cancer: From Tumor Progression to Resistance to Targeted Therapies.

Gelfo V, Romaniello D, Mazzeschi M, Sgarzi M, Grilli G, Morselli A, Manzan B, Rihawi K, Lauriola M.Int J Mol Sci. 2020 Aug 20;21(17): E6009.

A novel role for the interleukin-1 receptor axis in resistance to anti-EGFR therapy.

Valerio Gelfo, M. Mazzeschi, G. Grilli, M. Lindzen, S. Santi, G. D'Uva, B. Gyorffy, A. Ardizzoni, Y. Yarden and M. Lauriola. Cancers, September 2018

PD-L1 assessment on Circulating tumor Cells (CTCs) as prognostic biomarker in Advanced Non Small Cell Lung Cancer (A- NSCLC) patients treated with ImmuneCheckpointInhibitors (ICI)

Filippo G. Dall'Olio, Nicole Conci, Giulio Rossi, Michelangelo Fiorentino, Andrea De Giglio, Giada Grilli, Annalisa Altimari, Elisa Gruppioni, Daria M. Filippini, Alessandro Di Federico, Andrea Ardizzoni (ECLL 2019)

Molecular mechanisms of resistance to cetuximab mediated by IL1 signaling

Valerio Gelfo, G. Grilli, M. Mazzeschi, M. Lindzen, S. Santi, G. D'Uva, Y. Yarden and M. Lauriola. ABCD National Ph.D. Meeting. Salerno, Italia, 22-24 marzo 2018 – Abstract

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REFERENCES

1. Bade BC, Dela Cruz CS. Lung Cancer 2020: Epidemiology, Etiology, and Prevention. *Clin Chest Med*. 2020;41(1):1-24. doi:10.1016/j.ccm.2019.10.001

2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424. doi:10.3322/caac.21492

3. Murray CJL, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet*. 1997;349(9064):1498-1504. doi:10.1016/S0140-6736(96)07492-2

4. Torre LA, Siegel RL, Jemal A. Lung cancer statistics. *Adv Exp Med Biol*. 2016;893:1-19. doi:10.1007/978-3-319-24223-1_1

5. Gori S, Alavilla G, Ascierto P, et al. I numeri del cancro in Italia. *Rep AIOM-AIRTUM*. Published online 2020:1-232.

6. Chen Z, Li Z, Niu X, et al. The effect of CYP1A1 polymorphisms on the risk of lung

cancer: a global meta-analysis based on 71 case-control studies. *Mutagenesis*. 2011;26(3):437-446. doi:10.1093/MUTAGE/GER002

7. Amos CI, Wu X, Broderick P, et al. Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat Genet*. 2008;40(5):616-622. doi:10.1038/NG.109

8. Gazdar AF, Bunn PA, Minna JD. Small-cell lung cancer: what we know, what we need to know and the path forward. *Nat Rev Cancer 2017 1712*. 2017;17(12):725-737. doi:10.1038/nrc.2017.87

9. IARC. WORLD HEALTH ORGANIZATION INTERNATIONAL AGENCY FOR RESEARCH ON CANCER IARC Monographs on the Evaluation of Carcinogenic Risks to Humans VOLUME 83 Tobacco Smoke and Involuntary Smoking. *Tob Smoke Involunary Smok*. 2004;83:Volume 83.

10. Wieslander G, Norbäck D, Lindgren T. Experimental exposure to propylene glycol mist in aviation emergency training: acute ocular and respiratory effects. *Occup Environ Med*. 2001;58(10):649-655. doi:10.1136/OEM.58.10.649

11. Goniewicz ML, Knysak J, Gawron M, et al. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tob Control*. 2014;23(2):133-139. doi:10.1136/TOBACCOCONTROL-2012-050859

12. Paumgartten F. A critical appraisal of the harm reduction argument for heat-not-burn tobacco products. *Rev Panam Salud Pública*. 2018;42. doi:10.26633/RPSP.2018.161

13. Giuliani L, Jaxmar T, Casadio C, et al. Detection of oncogenic viruses SV40, BKV, JCV, HCMV, HPV and p53 codon 72 polymorphism in lung carcinoma. *Lung Cancer*.

2007;57(3):273-281. doi:10.1016/J.LUNGCAN.2007.02.019

14. Bobba RK, Holly JS, Loy T, Perry MC. Scar carcinoma of the lung: a historical perspective. *Clin Lung Cancer*. 2011;12(3):148-154. doi:10.1016/J.CLLC.2011.03.011

15. Travis WD, Brambilla E, Burke AP, al. et. WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart. Published online 2015.

16. Kuhn E, Morbini P, Cancellieri A, Damiani S, Cavazza A, Comin CE. Adenocarcinoma classification: Patterns and prognosis. *Pathologica*. 2018;110(1):5-11.

17. Robbins e Cotran. *Le Basi Patologiche Delle Malattie*. Vol 1. 1st ed.; 2015. Accessed January 21, 2022. https://www.amazon.it/Patologia-generale-Robbins-patologiche-malattie-ebook/dp/B01CDHJXM8/ref=sr_1_4?adgrpid=129648771486&gclid=CjwKCAiA0KmPBhBqEiwAJqKK4zZzH12TzPXc1AFhXeBrlS5s-GCvp6VxdDodRElw3zclQGm8-9vIDBoCsMEQAvD_BwE&hvadid=567901315128&hvdev=c&hvlocphy=1008141&hvnetw=g&hvqmt=b&hvrand=12493673243996657095&hvtargid=kwd-914479028135&hydadcr=28966_1805648&keywords=le+basi+patologiche+delle+malattie+2&qid=1642774340&sr=8-4

18. Travis WD. Pathology of lung cancer. *Clin Chest Med*. 2011;32(4):669-692. doi:10.1016/J.CCM.2011.08.005

19. Inamura K. Update on Immunohistochemistry for the Diagnosis of Lung Cancer. *Cancers (Basel)*. 2018;10(3). doi:10.3390/CANCERS10030072

20. Travis WD, Brambilla E, Nicholson AG, et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances

Since the 2004 Classification. *J Thorac Oncol*. 2015;10(9):1243-1260. doi:10.1097/JTO.0000000000000630

21. Xiaochuan L, Jiangyong Y, Ping Z, Xiaonan W, Lin L. Clinical characteristics and prognosis of pulmonary large cell carcinoma: A population-based retrospective study using SEER data. *Thorac cancer*. 2020;11(6):1522-1532. doi:10.1111/1759-7714.13420

22. Rekhtman N, Tafe LJ, Chaft JE, et al. Distinct profile of driver mutations and clinical features in immunomarker-defined subsets of pulmonary large-cell carcinoma. *Mod Pathol*. 2013;26(4):511-522. doi:10.1038/MODPATHOL.2012.195

23. Drilon A, Rekhtman N, Arcila M, et al. A Phase 2 Single Arm Trial of Cabozantinib in Patients with Advanced RET-Rearranged Lung Cancers. *Lancet Oncol*. 2016;17(12):1653. doi:10.1016/S1470-2045(16)30562-9

24. Lugo R, Gabasa M, Andriani F, et al. Heterotypic paracrine signaling drives fibroblast senescence and tumor progression of large cell carcinoma of the lung. *Oncotarget*. 2016;7(50):82324-82337. doi:10.18632/oncotarget.10327

25. Gabasa M, Radisky ES, Ikemori R, et al. MMP1 drives tumor progression in large cell carcinoma of the lung through fibroblast senescence. *Cancer Lett*. 2021;507:1-12. doi:10.1016/J.CANLET.2021.01.028

26. Kalhor N. Colloid carcinoma of the lung: Current views. *Semin Diagn Pathol*. 2014;31(4):265-270. doi:10.1053/J.SEMDP.2014.06.003

27. Ogusu S, Takahashi K, Hirakawa H, et al. Primary Pulmonary Colloid Adenocarcinoma: How Can We Obtain a Precise Diagnosis? *Intern Med*. 2018;57(24):3637. doi:10.2169/INTERNALMEDICINE.1153-18

28. Kradin RL, Young RH, Dickersin GR, Kirkham SE, Mark EJ. Pulmonary blastoma with argyrophil cells and lacking sarcomatous features (pulmonary endodermal tumor resembling fetal lung). *Am J Surg Pathol*. 1982;6(2):165-172. doi:10.1097/00000478-198203000-00009

29. Zhang J, Sun J, Liang XL, Lu JL, Luo YF, Liang ZY. Differences between low and high grade fetal adenocarcinoma of the lung: a clinicopathological and molecular study. *J Thorac Dis*. 2017;9(7):2071-2078. doi:10.21037/JTD.2017.07.14

30. Ricaurte LM, Arrieta O, Zatarain-Barrón ZL, Cardona AF. Comprehensive review of fetal adenocarcinoma of the lung. *Lung Cancer Targets Ther*. 2018;9:57-63. doi:10.2147/LCTT.S137410

31. Rossi G, Mengoli MC, Cavazza A, Al. E. Large cell carcinoma of the lung: clinically oriented classification integrating immunohistochemistry and molecular biology. *Virchows Arch*. 2014;464:61-68.

32. Antoine M, Vieira T, Fallet V, et al. Carcinomes sarcomatoïdes pulmonaires. *Ann Pathol*. 2016;36(1):44-54. doi:10.1016/j.annpat.2015.11.007

33. Domblides C, Leroy K, Monnet I, et al. Efficacy of Immune Checkpoint Inhibitors in Lung Sarcomatoid Carcinoma. *J Thorac Oncol*. 2020;15(5):860-866. doi:10.1016/J.JTHO.2020.01.014

34. WebPathology. LungAdenoCa:Giant Cells. https://www.webpathology.com/image.asp?case=416&n=128

35. Puzyrenko A, Shponka V, Sheinin Y, Giorgadze T, Nomani L. Primary pulmonary mucoepidermoid carcinoma: Cyto-histologic correlation and review of the literature. *Ann Diagn Pathol*. 2021;51(January):151698.

doi:10.1016/j.anndiagpath.2020.151698

36. Luo DL, Yan JH, Ge Y, et al. [Diagnostic and prognostic implications of MAML2 gene translocation in primary pulmonary mucoepidermoid carcinoma]. *Zhonghua bing li xue za zhi = Chinese J Pathol*. 2019;48(1):26-30. doi:10.3760/CMA.J.ISSN.0529-5807.2019.01.006

37. Moran, Cesar A. , Michael N. Koss SS. Primary adenoid cystic carcinoma of the lung. A clinicopathologic and immunohistochemical study of 16 cases. *Cancer*. 1994;73(1994):38-59. doi:10.1002/1097-0142(19940301)73

38. Högerle BA, Lasitschka F, Muley T, et al. Primary adenoid cystic carcinoma of the trachea: clinical outcome of 38 patients after interdisciplinary treatment in a single institution. *Radiat Oncol*. 2019;14(1). doi:10.1186/S13014-019-1323-Z

39. Hu MM, Hu Y, He JB, Li BL. Primary adenoid cystic carcinoma of the lung: Clinicopathological features, treatment and results. *Oncol Lett*. 2015;9(3):1475. doi:10.3892/OL.2015.2859

40. Stenman G, Persson F, Andersson MK. Diagnostic and therapeutic implications of new molecular biomarkers in salivary gland cancers. *Oral Oncol*. 2014;50(8):683-690. doi:10.1016/J.ORALONCOLOGY.2014.04.008

41. Wolkow N, Jakobiec FA, Afrogheh AH, et al. PD-L1 and PD-L2 Expression Levels Are Low in Primary and Secondary Adenoid Cystic Carcinomas of the Orbit: Therapeutic Implications. *Ophthal Plast Reconstr Surg*. 2020;36(5):444. doi:10.1097/IOP.0000000000001585

42. Bhattacharyya T, Bahl A, Kapoor R, Bal A, Das A, Sharma SC. Primary adenoid cystic carcinoma of lung: A case report and review of the literature. *J Cancer Res Ther*.

9(2). doi:10.4103/0973-1482.113399

43. Nakashima Y, Morita R, Ui A, Iihara K, Yazawa T. Epithelial-myoepithelial carcinoma of the lung: a case report. *Surg Case Reports*. 2018;4(1). doi:10.1186/S40792-018-0482-8

44. Zhang JY, Xu XY, Shen Q, Wang YF, Wang X, Zhou XJ. [Large-cell lung carcinoma by the 2015 WHO classification: a clinicopathologic analysis of 93 cases]. *Zhonghua bing li xue za zhi = Chinese J Pathol*. 2016;45(4):231-236. doi:10.3760/CMA.J.ISSN.0529-5807.2016.04.004

45. Nakaguro M, Nagao T. Epithelial–Myoepithelial Carcinoma. *Surg Pathol Clin*. 2021;14(1):97-109. doi:10.1016/j.path.2020.10.002

46. Patterson DT, Halverson Q, Williams S, Bishop JA, Ochoa CD, Styrvoky K. Bronchoscopic management of a primary endobronchial salivary epithelial-myoepithelial carcinoma: A case report. *Respir Med Case Reports*. 2020;30(May):101083. doi:10.1016/j.rmcr.2020.101083

47. Seethala RR, Chiosea SI. MAML2 Status in Mucoepidermoid Carcinoma Can No Longer Be Considered a Prognostic Marker. *Am J Surg Pathol*. 2016;40(8):1151-1153. doi:10.1097/PAS.0000000000000676

48. Seethala RR, Dacic S, Cieply K, Kelly LM, Nikiforova MN. A reappraisal of the MECT1/MAML2 translocation in salivary mucoepidermoid carcinomas. *Am J Surg Pathol*. 2010;34(8):1106-1121. doi:10.1097/PAS.0B013E3181DE3021

49. Behboundi A, Stenman G. Molecular Classification of Mucoepidermoid Carcinomas—Prognostic Significance of the MECT1–MAML2 Fusion Oncogene. *Genes Chromosomes Cancer*. 2006;54:470-481. doi:10.1002/gcc

50. Bundele M, Weinreb I, Xu B, et al. Mucoacinar Carcinoma: A Rare Variant of Mucoepidermoid Carcinoma. *Am J Surg Pathol*. 2021;45(8):1028-1037. doi:10.1097/PAS.0000000000001752

51. Seethala RR, Leon Barnes E, Hunt JL. Epithelial-myoepithelial carcinoma: a review of the clinicopathologic spectrum and immunophenotypic characteristics in 61 tumors of the salivary glands and upper aerodigestive tract. *Am J Surg Pathol*. 2007;31(1):44-57. doi:10.1097/01.PAS.0000213314.74423.D8

52. Yanagawa N, Sato A, Nishiya M, et al. Pulmonary epithelial-myoepithelial carcinoma without AKT1, HRAS or PIK3CA mutations: A case report. *Diagn Pathol*. 2020;15(1):1-5. doi:10.1186/S13000-020-01020-Z/TABLES/1

53. Osmani L, Askin F, Gabrielson E, Li QK. Current WHO guidelines and the critical role of immunohistochemical markers in the subclassification of non-small cell lung carcinoma (NSCLC): Moving from targeted therapy to immunotherapy. *Semin Cancer Biol*. 2018;52(Pt 1):103-109. doi:10.1016/j.semcancer.2017.11.019

54. Bégin LR, Eskandari J, Joncas J, Panasci L. Epstein-Barr virus related lymphoepithelioma-like carcinoma of lung. *J Surg Oncol*. 1987;36(4):280-283. doi:10.1002/JSO.2930360413

55. Liang Y, Wang L, Zhu Y, et al. Primary pulmonary lymphoepithelioma-like carcinoma: Fifty-Two patients with long-Term follow-up. *Cancer*. 2012;118(19):4748-4758. doi:10.1002/cncr.27452

56. Jha NK, Thomson BMK, Meredith D, Stubberfield J. Primary lymphoepithelioma-like carcinoma of lung. *Asian Cardiovasc Thorac Ann*. 2002;10(2):186-188. doi:10.1177/021849230201000226

57. Ho JC, Wong MP, Lam WK. Lymphoepithelioma-like carcinoma of the lung. *Respirology*. 2006;11(5):539-545. doi:10.1111/J.1440-1843.2006.00910.X

58. Xie Z, Liu L, Lin X, et al. A multicenter analysis of genomic profiles and PD-L1 expression of primary lymphoepithelioma-like carcinoma of the lung. *Mod Pathol*. 2020;33(4):626-638. doi:10.1038/S41379-019-0391-9

59. Chen B, Chen X, Zhou P, et al. Primary pulmonary lymphoepithelioma-like carcinoma: A rare type of lung cancer with a favorable outcome in comparison to squamous carcinoma. *Respir Res*. 2019;20(1):1-11. doi:10.1186/S12931-019-1236-2/TABLES/3

60. Mao N, Liao Z, Wu J, et al. Diagnosis of NUT carcinoma of lung origin by next-generation sequencing: case report and review of the literature. *Cancer Biol Ther*. 2019;20(2):150-156. doi:10.1080/15384047.2018.1523852

61. Jung M, Kim S, Lee J, et al. Clinicopathological and Preclinical Findings of NUT Carcinoma: A Multicenter Study. *Oncologist*. 2019;24(8). doi:10.1634/THEONCOLOGIST.2018-0477

62. Giridhar P, Mallick S, Kashyap L, Rath GK. Patterns of care and impact of prognostic factors in the outcome of NUT midline carcinoma: a systematic review and individual patient data analysis of 119 cases. *Eur Arch Otorhinolaryngol*. 2018;275(3):815-821. doi:10.1007/S00405-018-4882-Y

63. Chatzopoulos K, Boland JM. Update on genetically defined lung neoplasms: NUT carcinoma and thoracic SMARCA4-deficient undifferentiated tumors. *Virchows Arch*. 2021;478(1):21-30. doi:10.1007/S00428-020-03011-3

64. Liu S, Ferzli G. NUT carcinoma: a rare and devastating neoplasm. *Case Reports*.

2018;2018:bcr-2018-226526. doi:10.1136/BCR-2018-226526

65. Jones GS, Baldwin DR. Recent advances in the management of lung cancer. *Clin Med*. 2018;18(Suppl 2):s41-s46. doi:10.7861/CLINMEDICINE.18-2-S41

66. Westeel V. Diagnosis of lung cancer. *Rev Prat*. 2003;53(7):727-734. doi:10.4046/trd.1990.37.4.337

67. Lim W, Ridge CA, Nicholson AG, Mirsadraee S. The 8th lung cancer TNM classification and clinical staging system: review of the changes and clinical implications. *Quant Imaging Med Surg*. 2018;8(7):709. doi:10.21037/QIMS.2018.08.02

68. L K, F S. Lung Cancer Staging. *Surg Pathol Clin*. 2020;13(1):57-71. doi:10.1016/J.PATH.2019.10.003

69. Tsim S, O'Dowd CA, Milroy R, Davidson S. Staging of non-small cell lung cancer (NSCLC): A review. *Respir Med*. 2010;104(12):1767-1774. doi:10.1016/J.RMED.2010.08.005

70. Park JO, Lee S Il, Song SY, et al. Measuring response in solid tumors: comparison of RECIST and WHO response criteria. *Jpn J Clin Oncol*. 2003;33(10):533-537. doi:10.1093/JJCO/HYG093

71. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45(2):228-247. doi:10.1016/J.EJCA.2008.10.026

72. Ribas A, Chmielowski B, Glaspy JA. Do we need a different set of response assessment criteria for tumor immunotherapy? *Clin Cancer Res*. 2009;15(23):7116-7118. doi:10.1158/1078-0432.CCR-09-2376

73. Wolchok JD, Hoos A, O'Day S, et al. Guidelines for the Evaluation of Immune Therapy Activity in Solid Tumors: Immune-Related Response Criteria. *Clin Cancer Res*. 2009;15(23):7412-7420. doi:10.1158/1078-0432.CCR-09-1624

74. Mulkey F, Theoret MR, Keegan P, Pazdur R, Sridhara R. Comparison of iRECIST versus RECIST V.1.1 in patients treated with an anti-PD-1 or PD-L1 antibody: Pooled FDA analysis. *J Immunother Cancer*. 2020;8(1):1-7. doi:10.1136/jitc-2019-000146

75. Seymour L, Bogaerts J, Perrone A, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *Lancet Oncol*. 2017;18(3):e143-e152. doi:10.1016/S1470-2045(17)30074-8

76. Silvia Novello Francesco. Linee guida NEOPLASIE DEL POLMONE Edizione 2020. *AIOM*.

77. Barlesi F, Mazieres J, Merlio J-P, et al. Routine molecular profiling of patients with advanced non-small-cell lung cancer: results of a 1-year nationwide programme of the French Cooperative Thoracic Intergroup (IFCT). *Lancet*. 2016;387(10026):1415-1426. doi:10.1016/S0140-6736(16)00004-0

78. FDA. The Best Resource : Harmonizing Biomarker Terminology. *https://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/UCM510443.pdf*. 2016;(June). https://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/UCM510443.pdf

79. Nicholson R., Gee JM., Harper M. EGFR and cancer prognosis. *Eur J Cancer*. 2001;37:9-15. doi:10.1016/S0959-8049(01)00231-3

80. Brand TM, Iida M, Li C, Wheeler DL. The nuclear epidermal growth factor receptor

signaling network and its role in cancer. *Discov Med*. 2011;12(66):419-432. Accessed September 23, 2017. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3305885/pdf/nihms358983.pdf

81. Riely GJ, Politi KA, Miller VA, Pao W. Update on epidermal growth factor receptor mutations in non-small cell lung cancer. *Clin Cancer Res*. 2006;12(24):7232-7241. doi:10.1158/1078-0432.CCR-06-0658

82. Cemmi F, Stella GM, Zorzetto M, et al. Profilo mutazionale degli oncogeni EGFR e KRAS in cellule di carcinoma broncogeno non a piccole cellule ottenute da fine needle aspiration cytology (FNAC). *Boll della Soc Med Chir di Pavia*. 2010;123(2):231-281.

83. Kwak EL, Bang Y-J, Camidge DR, et al. Anaplastic Lymphoma Kinase Inhibition in Non–Small-Cell Lung Cancer. *N Engl J Med*. 2010;363(18):1693-1703. doi:10.1056/NEJMoa1006448

84. Toyokawa G, Seto T. Anaplastic lymphoma kinase rearrangement in lung cancer: its biological and clinical significance. *Respir Investig*. 2014;52(6):330-338. doi:10.1016/j.resinv.2014.06.005

85. Zhu Q, Zhan P, Zhang X, Lv T, Song Y. Clinicopathologic characteristics of patients with ROS1 fusion gene in non-small cell lung cancer: a meta-analysis. *Transl lung cancer Res*. 2015;4(3):300-309. doi:10.3978/j.issn.2218-6751.2015.05.01

86. Kim-Son H Nguyen, Susumu Kobayashi and DBC. Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancers dependent on the epidermal growth factor receptor pathway. *Clin Lung Cancer*. 2010;10(4):281-289. doi:10.3816/CLC.2009.n.039.Acquired

87. Zhou X, Wang X, Zhu H, et al. PI3K inhibition sensitizes EGFR wild-type NSCLC cell lines to erlotinib chemotherapy. *Exp Ther Med*. 2021;21(1):1-1. doi:10.3892/ETM.2020.9441

88. Novello Si, Passiglia F, Facchinetti F, et al. Linee Guida AIOM - NEOPLASIE DEL POLMONE, 2020. Published online 2020.

89. Mosele F, Remon J, Mateo J, et al. Recommendations for the use of next-generation sequencing (NGS) for patients with metastatic cancers: a report from the ESMO Precision Medicine Working Group. *Ann Oncol*. 2020;31(11):1491-1505. doi:10.1016/j.annonc.2020.07.014

90. Mateo J, Chakravarty D, Dienstmann R, et al. A framework to rank genomic alterations as targets for cancer precision medicine: The ESMO Scale for Clinical Actionability of molecular Targets (ESCAT). *Ann Oncol*. 2018;29(9):1895-1902. doi:10.1093/annonc/mdy263

91. JG T, S B, HC J, et al. COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res*. 2019;47(D1):D941-D947. doi:10.1093/NAR/GKY1015

92. Del T, Retto C. Linee guida. Published online 2015.

93. Lazzari C, Bulotta A, Cangi MG, et al. Next generation sequencing in non-small cell lung cancer: Pitfalls and opportunities. *Diagnostics*. 2020;10(12):1-12. doi:10.3390/diagnostics10121092

94. Samorodnitsky E, Jewell BM, Hagopian R, et al. Evaluation of Hybridization Capture Versus Amplicon-Based Methods for Whole-Exome Sequencing. *Hum Mutat*. 2015;36(9):903. doi:10.1002/HUMU.22825

95. Li W, Qiu T, Ling Y, Gao S, Ying J. Subjecting appropriate lung adenocarcinoma

samples to next-generation sequencing-based molecular testing: challenges and possible solutions. *Mol Oncol*. 2018;12(5):677-689. doi:10.1002/1878-0261.12190

96. Zhang YC, Zhou Q, Wu YL. The emerging roles of NGS-based liquid biopsy in non-small cell lung cancer. *J Hematol Oncol*. 2017;10(1). doi:10.1186/S13045-017-0536-6

97. Zill OA, Mortimer S, Banks KC, et al. Somatic genomic landscape of over 15,000 patients with advanced-stage cancer from clinical next-generation sequencing analysis of circulating tumor DNA. *https://doi.org/101200/JCO20163418_supplLBA11501*. 2016;34(18_suppl):LBA11501-LBA11501. doi:10.1200/JCO.2016.34.18_SUPPL.LBA11501

98. Fernandez-Gutierrez F, Foy V, Burns K, et al. OA06.01 Clinical Utility of Circulating Tumor DNA (ctDNA) Analysis by Digital next Generation Sequencing of over 5,000 Advanced NSCLC Patients. *J Thorac Oncol*. 2017;12(1):S263-S264. doi:10.1016/J.JTHO.2016.11.256

99. Lettig L, Sahnane N, Pepe F, et al. EGFR T790M detection rate in lung adenocarcinomas at baseline using droplet digital PCR and validation by ultra-deep next generation sequencing. *Transl Lung Cancer Res*. 2019;8(5):584. doi:10.21037/TLCR.2019.09.18

100. Vukmirovic M, Herazo-Maya JD, Blackmon J, et al. Identification and validation of differentially expressed transcripts by RNA-sequencing of formalin-fixed, paraffin-embedded (FFPE) lung tissue from patients with Idiopathic Pulmonary Fibrosis. *BMC Pulm Med*. 2017;17(1). doi:10.1186/S12890-016-0356-4

101. Evangelista AF, Zanon MF, Carloni AC, et al. Detection of ALK fusion transcripts in FFPE lung cancer samples by NanoString technology. *BMC Pulm Med*. 2017;17(1). doi:10.1186/S12890-017-0428-0

102. Akahori D, Inoue Y, Inui N, et al. Comparative assessment of NOIR-SS and ddPCR for ctDNA detection of EGFR L858R mutations in advanced L858R-positive lung adenocarcinomas. *Sci Rep*. 2021;11(1). doi:10.1038/S41598-021-94592-9

103. Pennell NA, Mutebi A, Zhou Z-Y, et al. Economic impact of next generation sequencing vs sequential single-gene testing modalities to detect genomic alterations in metastatic non-small cell lung cancer using a decision analytic model. *J Clin Oncol*. 2018;36(15_suppl):9031-9031. doi:10.1200/JCO.2018.36.15_suppl.9031

104. J Z, R C, A B, G Z. The impact of next-generation sequencing on genomics. *J Genet Genomics*. 2011;38(3):95-109. doi:10.1016/J.JGG.2011.02.003

105. McCarthy EF. The Toxins of William B. Coley and the Treatment of Bone and Soft-Tissue Sarcomas. *Iowa Orthop J*. 2006;26:154. Accessed December 28, 2021. /pmc/articles/PMC1888599/

106. Hinshaw DC, Shevde LA. The Tumor Microenvironment Innately Modulates Cancer Progression. *Cancer Res*. 2019;79(18):4557-4567. doi:10.1158/0008-5472.CAN-18-3962

107. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*. 2004;21(2):137-148. doi:10.1016/J.IMMUNI.2004.07.017

108. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*.

2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013

109. Bretscher PA. A two-step, two-signal model for the primary activation of precursor helper T cells. *Proc Natl Acad Sci U S A*. 1999;96(1):185-190. doi:10.1073/PNAS.96.1.185

110. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 SYSTEM OF T CELL COSTIMULATION. *http://dx.doi.org/101146/annurev.immunol141233*. 2003;14:233-258. doi:10.1146/ANNUREV.IMMUNOL.14.1.233

111. MA O, RJ G, DA M, RB L, AH S. CTLA-4 and T cell activation. *Curr Opin Immunol*. 1999;11(3):294-300. doi:10.1016/S0952-7915(99)80047-8

112. Ranheim EA, Kipps TJ. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J Exp Med*. 1993;177(4):925. doi:10.1084/JEM.177.4.925

113. Koulova L, Clark EA, Shu G, Dupont B. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. *J Exp Med*. 1991;173(3):759-762. doi:10.1084/jem.173.3.759

114. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol*. 2016;39(1):98-106. doi:10.1097/COC.0000000000000239

115. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J*. 1992;11(11):3887. doi:10.1002/j.1460-2075.1992.tb05481.x

116. Ai L, Xu A, Xu J. Roles of PD-1/PD-L1 Pathway: Signaling, Cancer, and Beyond. *Adv Exp Med Biol*. 2020;1248:33-59. doi:10.1007/978-981-15-3266-5_3

117. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. *Am J Cancer Res*. 2020;10(3):727-742. Accessed December 29, 2021. https://pubmed.ncbi.nlm.nih.gov/32266087/

118. 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):123-135. doi:10.1056/NEJMOA1504627/SUPPL_FILE/NEJMOA1504627_DISCLOSURES.PDF

119. Saltz LB, Clarke S, Díaz-Rubio E, et al. Bevacizumab in Combination With Oxaliplatin-Based Chemotherapy As First-Line Therapy in Metastatic Colorectal Cancer: A Randomized Phase III Study. *J Clin Oncol*. 2008;26(12):2013-2019. doi:10.1200/JCO.2007.14.9930

120. Herbst RS, Baas P, Kim DW, 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):1540-1550. doi:10.1016/S0140-6736(15)01281-7

121. 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 (London, England)*. 2016;387(10030):1837-1846. doi:10.1016/S0140-6736(16)00587-0

122. 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):255-265. doi:10.1016/S0140-6736(16)32517-X

123. Hellmann MD, Rizvi NA, Goldman JW, et al. Nivolumab plus ipilimumab as first-line

treatment for advanced non-small-cell lung cancer (CheckMate 012): results of an open-label, phase 1, multicohort study. *Lancet Oncol*. 2017;18(1):31-41. doi:10.1016/S1470-2045(16)30624-6

124. 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):1823-1833. doi:10.1056/NEJMOA1606774/SUPPL_FILE/NEJMOA1606774_DISCLOSURES.PDF

125. Herbst RS, Giaccone G, de Marinis F, et al. Atezolizumab for First-Line Treatment of PD-L1–Selected Patients with NSCLC. *N Engl J Med*. 2020;383(14):1328-1339. doi:10.1056/NEJMOA1917346/SUPPL_FILE/NEJMOA1917346_DATA-SHARING.PDF

126. Zhang J, Wu D, Zhang Z, et al. Pembrolizumab or Bevacizumab Plus Chemotherapy as First-Line Treatment of Advanced Nonsquamous Nonsmall Cell Lung Cancer: A Retrospective Cohort Study. *Technol Cancer Res Treat*. 2021;20:153303382110396. doi:10.1177/15330338211039676

127. Hellmann MD, Paz-Ares L, Bernabe Caro R, et al. Nivolumab plus Ipilimumab in Advanced Non–Small-Cell Lung Cancer. *N Engl J Med*. 2019;381(21):2020-2031. doi:10.1056/NEJMOA1910231/SUPPL_FILE/NEJMOA1910231_DATA-SHARING.PDF

128. Oxnard GR, Yang JCH, Yu H, et al. TATTON: a multi-arm, phase Ib trial of osimertinib combined with selumetinib, savolitinib, or durvalumab in EGFR-mutant lung cancer. *Ann Oncol Off J Eur Soc Med Oncol*. 2020;31(4):507-516. doi:10.1016/J.ANNONC.2020.01.013

129. Agrawal T, Artis E, Xie J, et al. P76.74 PAPILLON: Randomized Phase 3 Study of Amivantamab Plus Chemotherapy vs Chemotherapy Alone in EGFR Exon20ins NSCLC. *J Thorac Oncol*. 2021;16(3):S621. doi:10.1016/J.JTHO.2021.01.1131

130. An Open-label Study of Encorafenib + Binimetinib in Patients With BRAFV600-mutant Non-small Cell Lung Cancer - Full Text View - ClinicalTrials.gov. Accessed November 5, 2021. https://clinicaltrials.gov/ct2/show/NCT03915951

131. An Open-label Study of Encorafenib + Binimetinib in Patients With BRAFV600-mutant Non-small Cell Lung Cancer - National Cancer Institute. Accessed November 5, 2021. https://www.cancer.gov/about-cancer/treatment/clinical-trials/search/v?id=NCI-2019-02644&r=1

132. A D, GR O, DSW T, et al. Efficacy of Selpercatinib in RET Fusion-Positive Non-Small-Cell Lung Cancer. *N Engl J Med*. 2020;383(9):813-824. doi:10.1056/NEJMOA2005653

133. Markham A. Selpercatinib: First Approval. *Drugs*. 2020;80(11):1119-1124. doi:10.1007/S40265-020-01343-7

134. Solomon BJ, Zhou CC, Drilon A, et al. Phase III study of selpercatinib versus chemotherapy ± pembrolizumab in untreated RET positive non-small-cell lung cancer. *Futur Oncol*. 2021;17(7):763-773. doi:10.2217/FON-2020-0935

135. Hong L, Zhang J, Heymach J V., Le X. Current and future treatment options for MET exon 14 skipping alterations in non-small cell lung cancer: *https://doi.org/101177/1758835921992976*. 2021;13. doi:10.1177/1758835921992976

136. Drilon A, Laetsch TW, Kummar S, et al. Efficacy of Larotrectinib in TRK Fusion–

Positive Cancers in Adults and Children. *http://dx.doi.org/101056/NEJMoa1714448*. 2018;378(8):731-739. doi:10.1056/NEJMOA1714448

137. Dall'Olio FG, Conci N, Rossi G, et al. Comparison of Sequential Testing and Next Generation Sequencing in advanced Lung Adenocarcinoma patients – A single centre experience. *Lung Cancer*. 2020;149:5-9. doi:10.1016/j.lungcan.2020.08.008

138. Wang L, Hu H, Pan Y, et al. PIK3CA Mutations Frequently Coexist with EGFR/KRAS Mutations in Non-Small Cell Lung Cancer and Suggest Poor Prognosis in EGFR/KRAS Wildtype Subgroup. *PLoS One*. 2014;9(2):e88291. doi:10.1371/JOURNAL.PONE.0088291

139. K R, R A, M F, et al. MYC Amplification as a Potential Mechanism of Primary Resistance to Crizotinib in ALK-Rearranged Non-Small Cell Lung Cancer: A Brief Report. *Transl Oncol*. 2019;12(1):116-121. doi:10.1016/J.TRANON.2018.09.013

140. A S, L W, M H, et al. c-Myc alterations confer therapeutic response and acquired resistance to c-Met inhibitors in MET-addicted cancers. *Cancer Res*. 2015;75(21):4548-4559. doi:10.1158/0008-5472.CAN-14-2743

141. RM K, NM S, CH W, et al. Myc Cooperates with Ras by Programming Inflammation and Immune Suppression. *Cell*. 2017;171(6):1301-1315.e14. doi:10.1016/J.CELL.2017.11.013

142. Fukazawa T, Maeda Y, Matsuoka J, et al. Inhibition of myc effectively targets KRAS mutation-positive lung cancer expressing high levels of Myc. *Anticancer Res*. 2010;30(10):4193-4200.

143. Alves CL, Ehmsen S, Terp MG, et al. Co-targeting CDK4/6 and AKT with endocrine therapy prevents progression in CDK4/6 inhibitor and endocrine therapy-resistant breast cancer. *Nat Commun 2021 121*. 2021;12(1):1-15. doi:10.1038/s41467-021-25422-9

144. Monica S La, Fumarola C, Cretella D, et al. EGFR-Mutated NSCLC Cell Lines with Different Resistance Mechanisms to Osimertinib. Published online 2021.

145. Bouclier C, Simon M, Laconde G, et al. Stapled peptide targeting the CDK4/Cyclin D interface combined with Abemaciclib inhibits KRAS mutant lung cancer growth. *Theranostics*. 2020;10(5):2008-2028. doi:10.7150/THNO.40971

146. Santana-Davila R, Chow LQ. The use of combination immunotherapies as front-line therapy for non-small-cell lung cancer. *Futur Oncol*. 2018;14(3):191-194. doi:10.2217/fon-2017-0124

147. Socinski MA, Jotte RM, Cappuzzo F, et al. Atezolizumab for First-Line Treatment of Metastatic Nonsquamous NSCLC. *N Engl J Med*. 2018;378(24):2288-2301. doi:10.1056/nejmoa1716948

148. Gelsomino F, Lamberti G, Tiseo M, et al. Atezolizumab in a CoHort of pretreated, advanced, non-small cell lung cancer patients with rare HistologiCal SubtypEs (CHANCE trial). *Ther Adv Med Oncol*. 2020;12. doi:10.1177/1758835920915983

149. GOIRC. Atezolizumab in Advanced Non-small Cell Lung Cancer With Rare Histologies (CHANCE Trial) (CHANCE). Clinicaltrials.gov. Published 2018. https://clinicaltrials.gov/ct2/show/NCT03976518

150. AIFA. Gestione degli studi clinici in Italia in corso di emergenza COVID-19 (coronavirus disease 19). *AIFA*. 2020;ver2(1-12):43-54.

151. Kim C, Rajan A, DeBrito PA, Giaccone G. Metastatic lymphoepithelioma-like carcinoma of the lung treated with nivolumab: A case report and focused review of literature. *Transl Lung Cancer Res*. 2016;5(6):720-726. doi:10.21037/tlcr.2016.11.06

152. Salati M, Baldessari C, Calabrese F, et al. Nivolumab-Induced Impressive Response of Refractory Pulmonary Sarcomatoid Carcinoma with Brain Metastasis. *Case Rep Oncol*. 2018;11(3):615-621. doi:10.1159/000492666

153. Kumar V, Dave V, Harris J, Huang Y. Response of advanced stage recurrent lymphoepithelioma-like carcinoma to nivolumab. *Immunotherapy*. 2017;9(12):955-961. doi:10.2217/imt-2017-0067

154. Patel SP, Megan Othus M, Young Kwang C, Plets M, D. Blanke C, Kurzrock R. A Phase II Basket Trial of Dual Anti-CTLA-4 and Anti-PD-1 Blockade in Rare Tumors (DART SWOG 1609) in Patients with Non-Pancreatic Neuroendocrine Tumors. *Physiol Behav*. 2020;26(10):2290-2296. doi:10.1158/1078-0432.CCR-19-3356. A

155. Wang R, Hu H, Pan Y, et al. RET fusions define a unique molecular and clinicopathologic subtype of non-small-cell lung cancer. *J Clin Oncol*. 2012;30(35):4352-4359. doi:10.1200/JCO.2012.44.1477

156. Drilon A, Oxnard GR, Tan DSW, et al. Efficacy of Selpercatinib in RET Fusion-Positive Non-Small-Cell Lung Cancer. *N Engl J Med*. 2020;383(9):813-824. doi:10.1056/NEJMOA2005653

157. Plaza-Menacho I, Mologni L, McDonald NQ. Mechanisms of RET signaling in cancer: Current and future implications for targeted therapy. *Cell Signal*. 2014;26(8):1743-1752. doi:10.1016/j.cellsig.2014.03.032

158. Drilon A, Rekhtman N, Arcila M, et al. Cabozantinib in patients with advanced RET-rearranged non-small-cell lung cancer: an open-label, single-centre, phase 2, single-arm trial. *Lancet Oncol*. 2016;17(12):1653-1660. doi:10.1016/S1470-2045(16)30562-9

159. Gainor JF, Curigliano G, Kim DW, et al. Pralsetinib for RET fusion-positive non-small-cell lung cancer (ARROW): a multi-cohort, open-label, phase 1/2 study. *Lancet Oncol*. 2021;22(7):959-969. doi:10.1016/S1470-2045(21)00247-3