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# PROFILO GENETICO-MOLECOLARE DI ADENOCARCINOMI POLMONARI DURANTE LA PANDEMIA COVID-19

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# 1. INTRODUCTION

## 1.1 Lung Cancer Background

Lung cancer is the most often diagnosed cancer in the world and the most frequent cause of cancer death. The prognosis for lung cancer is relatively poor and 75% of patients are diagnosed at its advanced stage. The currently used diagnostic tools are not sensitive enough to diagnose it at early stage of the disease. Therefore, searching for new methods of early and accurate diagnosis of lung cancer is crucial for its effective treatment. Lung cancer is the result of multistage carcinogenesis with gradually increasing genetic and epigenetic changes. Screening for the characteristic genetic markers could enable the diagnosis of lung cancer at its early stage.[1]

In 2012, approximately 1.6 million people died of lung cancer and it is estimated that the number of lung cancer deaths will increase to 3 million in 2035.[2,3] Lung cancer has a relatively poor prognosis, and the 5-year survival varies from 4% to 17%, depending on the stage of the disease at the time of its diagnosis.[4] The advancement of non-invasive diagnostics enhances the possibility of detecting lung cancer, however only 10-15% of new cases are diagnosed at its clinical early stage.[5] Nevertheless, in patients with clinical stage IA disease in the tumor-lymph nodes-metastasis (TNM) classification, the 5-year survival reaches approximately 60%, which indicates that a large number of patients suffer from undetectable metastases at this stage of the disease.[6-8] The currently used diagnostic tools (i.e. chest radiography and sputum cytology) are not sensitive enough in the diagnosis of non-small cell lung carcinoma (NSCLC), while tumor markers, such as carcinoembryonic antigen (CEA), CYFRA 21-1, neuron-specific enolase (NSE), or squamous cell carcinoma antigen (SCCA) do not make the diagnosis possible at the early stage of lung cancer.[5] These data indicate the need to find more specific, less invasive biomarkers that could be used alternatively or complementary to radiological approaches and improve lung cancer detection and the determination of its stage.[9] Lung cancer does not result from the sudden transformation of bronchia epithelioma but from the final stage of multistage carcinogenesis, with gradually increasing genetic and epigenetic changes.[8,10] The main etiological factor is the exposure to the carcinogenic components of tobacco smoke. About 90% of lung cancer cases in men and 80% in women are caused by smoking. Nowadays, mutations that are characteristic of lung cancer and which may enable diagnosis at the early stage of the disease are searched for. The advancement of molecular strategies and analytic platforms makes possible to analyze the genome changes leading to cancer development (i.e. the potential biomarkers of lung cancer). In the reviewed studies, the

diagnostic values of microsatellite changes, DNA hypermethylation, Tumor Protein P53 (*TP53*) and V-Ki-Ras2 Kirsten Rat Sarcoma 2 Viral Oncogene Homolog (*KRAS*) gene mutations, as well as micro-RNA (miRNAs) expression, have been analyzed as potential new genetic markers. It seems that miRNAs and their expression profiles have the greatest diagnostic potential value in lung cancer diagnosis, but their quantification requires standardization.[1]

## 1.2 Lung Cancer Screening and Clinical Trials

Screening for lung cancer using low-dose computed tomography (LDCT) has been established as an effective screening modality that reduces lung cancer mortality.[11-13] The United States Preventive Services Task Force (USPSTF) recommends lung cancer screening for current and former smokers aged between 55–80 years, with at least 30 pack-years smoking exposure and within 15 years from smoking cessation.[14] While simple and practical, this risk stratification of ever-smokers is suboptimal because it recommends lung cancer screening to individuals at a very low risk who are unlikely to benefit from screening and excludes individuals at high risk of developing lung cancer who are more likely to benefit from screening.[15,16] In Europe, although population-scale lung cancer screening programs have not been yet implemented, a well-cited position statement on lung cancer screening supports a risk-based approach for selecting individuals to undergo lung cancer screening.[17,18-21]

The National Lung Screening Trial (NLST) has shown that screening high-risk individuals for lung cancer with LDCT yields 20% lung cancer specific mortality reduction as well as a 6.7% reduction in overall mortality.[12] The Dutch-Belgian Lung Cancer Screening (NELSON) trial demonstrated an even higher lung cancer mortality reduction than NLST.[13] In the US, existing guidelines on lung cancer screening recommend annual screening for high-risk individuals.[14,22-25] To address this limitation of existing guidelines, several lung cancer risk prediction models have been developed in recent years.[17,26-28] Several risk factors have been associated with and used as predictors of lung cancer risk. The most dominant risk factors for lung cancer are smoking and age. Sex, race/ethnicity, family history of lung cancer, COPD, emphysema, and exposure to asbestos and radon are some additional risk factors associated with lung cancer. Upon the conclusion of the NLST, numerous lung cancer risk prediction models have been developed, demonstrating superior selection of individuals for screening as compared to existing guidelines. In fact, the National Comprehensive Cancer Network (NCCN) guidelines on lung cancer screening endorse utilization of a risk prediction model for identification of high risk individuals to supplement NLST's eligibility criteria,[23] and the USPSTF is currently considering risk-based screening as it revises its recommendations on lung cancer screening.[17,29] The goal was and continuing up to day, to focus

on the application of the risk prediction models rather than their development and providing an overview of a predictive performance and intended application.[30]

This topic has long been interest in attempting early lung cancer detection through screening, first with chest radiography and, beginning in the 1990s, with chest low-dose computed tomography (LDCT) scanning. Chest radiography was the subject of three trials supported by the National Cancer Institute (NCI) in the 1970s, with studies at Memorial Sloan-Kettering, Johns Hopkins, and the Mayo Clinic. The Sloan-Kettering and Johns Hopkins trials added sputum cytology every 4 months to an annual chest radiograph and compared that with annual chest radiograph alone; the Mayo trial compared annual chest radiograph and sputum cytology every 3 months. The Memorial Sloan-Kettering and Johns Hopkins studies showed no difference in mortality rate by adding sputum cytology to annual chest radiograph. The Mayo Clinic study did not show a difference in mortality between groups or a shift toward detection of earlier-stage cancers and lacked sufficient power to detect a mortality advantage.[31,32] Based on the finding from the NCI trials, in 1980 the American Cancer Society (ACS) changed policy and recommended against any mass screening tests for early detection of lung cancer,[33] and began to emphasize prevention and tobacco cessation. However, they did continue to support chest radiographs for heavy smokers and asbestos workers.[34] Physician behavior seemed hard to change-ACS surveys of cancer screening in 1984 and 1989 found 44% of primary care physicians (PCPs) in both surveys ordering chest radiographs for screening asymptomatic patients for lung cancer, well after the ACS and others had advised against its use. Men and women at 10 centers were seen between 1993 and 2001. There was no eligibility requirement concerning smoking. The intervention group had a baseline chest radiograph and annual radiographs for 3 more years. There were no effects on cumulative lung cancer mortality over the 13 years of observation in the trial or stage shift in lung cancer at diagnosis.[35] In the early 1990s, studies began using LDCT scan as a modality for lung cancer screening.[36-40]

Subsequent single-arm trials in Europe, Japan, and the United States affirmed that LDCT showed superiority over chest radiograph for lung cancer screening, finding approximately 4 times more tumors than chest radiograph but did not show that screening saved lives. The NLST, published in 2011, enrolled 454 participants over 5 years. Individuals were between ages 55 and 74 with a 30 pack-year history of smoking, and LDCT was compared with chest radiograph using annual examination for 3 years. Median follow-up time was 5.6 years. A total of 1060 lung cancers were found in the LDCT arm, compared with 941 in the chest radiograph group. Almost twice as many early-stage cancers were diagnosed with LDCT compared with chest radiograph (40% *versus* 21%, respectively). There was a 20% relative reduction in lung cancer mortality in the LDCT group; the

number needed to screen to prevent death was 320. This was the first trial to show a reduction in lung cancer-specific mortality through LDCT screening.[41]

Despite criticism about its false-positive results, cost, over-diagnosis, and patient anxiety, the NLST spurred the adoption of lung cancer screening guidelines. In 2012, when Wood and colleagues[42] wrote the Clinical Guidelines in Oncology for Lung Cancer Screening for the National Comprehensive Cancer Network (NCCN), that agrees with the NLST/US Preventive Services Task Force (USPSTF) criteria and has issued a recommendation for LDCT screening that is not confined to cigarette smoking but includes other factors, such as radon exposure, occupational exposure to carcinogens, family history of lung cancer, and a personal history of cancer or chronic lung disease. Adults with a less intense smoking history and none of the other listed risk factors are classified as lower risk, and LDCT screening for lung cancer is not recommended. Early in 2013, the ACS updated its lung cancer screening guideline and recommended the NLST eligibility criteria, similar to the NCCN.[42,43] Later in 2013, the USPSTF reviewed the NLST data and in 2013 issued a grade B recommendation supporting annual lung cancer screening with LDCT for asymptomatic individuals 55 years to 80 years of age with a 30 pack-year history of smoking who currently smoke or have quit within the past 15 years.[44,45] After publication of the NLST and the USPSTF recommendations, several other medical societies and health groups issued guidelines regarding lung cancer screening, most of which adhere closely to the USPSTF recommendations.

## 1.3 Overcoming of COVID-19 in Clinical Routine

Cancer patients are highly vulnerable to SARS-CoV-2 infections due to frequent contacts with the healthcare system, immunocompromised state from cancer or its therapies, supportive medications such as steroids and most importantly their advanced age and comorbidities. Patients with lung cancer have consistently been reported to suffer from an increased risk of death compared with other cancers. This is possibly due to the combination of specific pathophysiological aspects, including underlying pulmonary compromise due to smoking history and the increased specific pressures on respiratory healthcare services caused by the related pandemic. Deciding whether to offer, modify, post-pone or even delay treatments for this patients' population has become crucial. Chemotherapy, immunotherapy, and targeted agents represent distinct risks factors in the context of COVID-19, that should be balanced with the short- and long-term consequences of delaying cancer care. Despite rapid and persistent trend of the pandemic declared by World Health Organization (WHO), various efforts were made by oncologists worldwide to understand the impact of COVID-19 on patients with cancer. Adapted recommendations of evidence-based practice guidelines have been developed with different small and large-scale registries, such as the COVID-19 and Cancer

Consortium (CCC19) and Thoracic Cancers International COVID-19 Collaboration collected data, supporting cancer cares under the challenging circumstance created by the COVID-19 pandemic. Several recommendations were developed as guidance for prioritizing various aspects of lung cancer care and mitigate adverse effects of the COVID-19 healthcare crisis, potentially reducing the morbidity and mortality of patients from COVID-19 and from cancer. Data collected are heterogeneous due to patients' demographics, variable testing availability or policy, and cancer patients' management guidelines over time.[46,47] Available data confirm that patients with lung cancer are possibly more likely to be infected by SARS-CoV-2 and, more importantly, definitively prone to develop severe complications defined as admission in an intensive care unit. Mortality rate in patients with cancer has been reported to be 25–30%.[47-49] Large datasets demonstrate that cancer is independently associated with mortality in hospitalized patients with COVID-19. Moreover, not all cancers appear to have equal risks of morbidity and mortality, with a mortality range from 8% to 30%.[50,51]

## 1.4 Patients with lung cancer: severity of COVID-19

Lung cancer represents a clinical scenario characterized by an increased risk of pulmonary complications, severe lung injury and high mortality from COVID-19, due to pathophysiological, clinical and treatment-related risk factors.[52] An important interplay of factors frequently associated with lung cancer, including smoking-related lung damage, significant cardiovascular and respiratory comorbidities, older age, induce higher severity of SARS-CoV-2 infection.[52-54]

Patients with lung cancer show fragility related to defective pulmonary and alveolar architecture due to previous thoracic surgery or radiotherapy and/or severe airway obstruction that may also predispose to more infections. Alterations of alveolar epithelium and pulmonary vessels lead to tumor microenvironment modifications, including an increase in immune cells and tissue-resident macrophages infiltration, critical for innate immunity and inflammation.[47,55] This widespread immune response within alveolar epithelium poses a higher risk for cytokine release; the alveolar damage and fibrin deposits represent risk factors for thrombosis of pulmonary vessels ad micro-vessels. These two pathogenetic basis have been postulated to be the major steps in leading to the development of severe lung injury and acute respiratory distress syndrome (ARDS) during SARS-CoV-2 infection.[47,56] Modifications in peribronchiolar homeostasis and inflammation additionally impair the building of an efficient antiviral or bacterial immune response required for infection clearance.[57-59]

Moreover, smokers showed high risk (× 2.4 times) of COVID-19 severe events compared with non-smokers (RR=2.4, 95% CI 1.43 to 4.04).[59] A cumulative risk for severe viral infection injury is

related to long-term tobacco-related lung damage, including chronic obstructive pulmonary disease (COPD) and lung cancer.[60] Finally, all-cause case–fatality rate in patients with cancer after SARS-CoV-2 infection was significantly associated with increasing age, rising steadily from 65 years, encompassing the majority of patients with lung cancer.[61]

Interaction between these pathological and clinical factors, in addition to the complex role of antineoplastic therapy and concomitant medications, define patients with lung tumour as an extremely vulnerable patient subgroup at the time of COVID-19 pandemic, for severe illness.

In this regard Thoracic canceERs international COVID-19 cOLlaboraTion (TERAVOLT) has been established. A structured analysis of patients with lung cancer and viral infection was investigated in the TERAVOLT registry. This study enrolled patients with thoracic cancer (NSCLC, mesothelioma, thymic epithelial tumors and other pulmonary neuroendocrine neoplasms) with either confirmed COVID-19 infection or clinically diagnosed infection.[60] The updated results based on 1012 patients showed higher mortality (32%) and hospitalization rate (72%), compared with so many low ICU admission (12%) and mechanical ventilation rate (7%).[62]

TERAVOLT study confirmed viral infection related general risk factors for mortality, including age, comorbidities and cancer-specific factors, namely PS (≥2 *versus* <2), finally, use of steroids prior to COVID-19 diagnosis.[62,63]

Interesting data have been reported during the first peak of COVID-19 pandemic by the Veneto region (Northeast of Italy): investigators collected data from 84.246 consecutive Italians tested for SARS-CoV-2 to evaluate the prevalence of cancer and clinical outcomes of viral infection. Among the 9275 SARS-CoV-2 positive patients, 723 had a cancer diagnosis (7.8%), which was associated with a high rate of hospitalization (56.6 *versus* 34.4%) and mortality (14.7% *versus* 4.5%) particularly for males and elderly patients. NSCLC was associated with a fourfold risk of death due to SARS-CoV-2 infection, although the patient population was too small to draw robust conclusions. This is what has happened almost in all over Italy. It has been extended to European and World situation.[64]

## 1.5 Lung Cancer Treatment and COVID-19

Primary goals of the treatment in patients affected of lung cancer during the COVID-19 pandemic are to minimize the risk of patient exposure to COVID-19, while managing tumour, which has a high risk of mortality.[65] Early in the pandemic, when there were shortages of personal protective equipment, limited COVID-19 testing supplies; many regions of the world saw overwhelmed healthcare systems, lung cancer surgeries were delayed and systemic treatments were canceled. Fortunately, at the current time, additional data have been gathered from TERAVOLT, CCC-19,

and others, allowing providers and patients to make a risk–benefit analysis of proceeding with treatment versus delaying or omitting. Data from the main registries and series about the impact of specific cancer treatment for NSCLC during the pandemic have been analyzed. During these troubled times, different evidence-based recommendations [i.e, American Society of Clinical Oncology (ASCO),[66] European Society for Medical Oncology (ESMO)[67,68] and International Association for the Studyum of Lung Cancer (IASLC)][65] were developed to improve the management of patients with lung cancer and to mitigate the pandemic impact on our patients.

### *1.5.1. Radiation therapy*

The European Society for Radiotherapy and Oncology and the American Society for Radiation published 'Practice recommendations for lung cancer radiotherapy during the COVID-19 pandemic.[69] Treatment recommendations are given for a variety of clinical scenarios, from stage I–III NSCLC, prophylactic cranial irradiation for small cell lung cancer (SCLC), and palliative radiation for NSCLC. Recommendations are given for both an 'early pandemic scenario', where risk mitigation of both patient and radiotherapy staff are balanced with the treatment of lung cancer, as well as a later pandemic scenario, where resources are limited requiring patient triage. Raccomandations include hypofractionated radiation schedules to reduce the number of visits to heath care facilities, to avoid twice daily radiation the use of single fraction radiation in the palliative setting, and the delay or omission of prophylactic cranial irradiation for patients with SCLC.[70,71]

### *1.5.2. Immunotherapy*

Early in the pandemic, there was concern that immune checkpoint inhibitors (ICIs) may worsen the complications of COVID-19. The concern was that ICIs may increase the cytokine storm causing ARDS of COVID-19, as well as the possibility of overlapping pneumonitis from ICIs and COVID-19.[72] However, the results of multiple registries including TERAVOLT,[60-63] the CCC19,[51,73] as well as 102 patients with lung cancer treated at Memorial Sloan Kettering Cancer Center,[74,75] all demonstrated that patients treated with ICIs alone, without chemotherapy, had outcomes equivalent or better to those receiving other cancer treatments. Given this information, for patients who have high PDL1 expression ≥50%, treatment with immunotherapy alone should be strongly considered over chemoimmunotherapy.[65] This can limit both patient and provider exposures, while retaining efficacy, thereby optimizing the risk–benefit ratio.[65,68] Pembrolizumab 400 mg given every 6 weeks has been approved by both European Medicines Agency (EMA) and US Food and Drug Administration (FDA).[76] Nivolumab can be dosed 480 mg every 4 weeks as opposed to every

other week.[77] Atezolizumab is able to be dosed every 4 weeks at 1680 mg.[78] Durvalumab dosing of 1500 mg every 4 weeks was initially approved and has demonstrated efficacy in the small cell population;[79] every 4-week dosing can be considered for the stage III NSCLC consolidation setting as well.[65] Pneumonitis present on CT scans for patients with lung cancer has traditionally had a differential of immunotherapy pneumonitis, radiation pneumonitis, or progressive lung cancer depending on the clinical context. COVID-19 pneumonitis has also been reported on imaging and can be difficult to differentiate based on imaging alone. CT findings suggestive of COVID-19 should lead to an appropriate infectious workup for COVID-19 while taking in to account the remaining differential.[65,80]

### *1.5.3. Chemotherapy*

In the original TERAVOLT analysis of 200 patients, treatment with chemotherapy alone was associated with increased risk of death (HR 2.54, 95% CI 1.09 to 6.11).[60]

In an updated analysis of 1012 patients presented at ESMO, patients receiving chemotherapy alone or not receiving treatment had worse outcomes compared with patients receiving immunotherapy, chemoimmunotherapy, or targeted therapy (HR 1.4, 95% CI 1.02 to 2.0, p=0.03).[62]

In a single center analysis of 102 patients with lung cancer treated at Memorial Sloan Kettering, no effect of recent systemic therapy was seen on COVID-19 outcomes, including chemotherapy or chemoimmunotherapy.[51-74] Patients receiving chemotherapy or chemoradiation had a 30-day mortality of 18% (Wise-Draper ESMO).[81] However, it is difficult to know how to interpret these data given the inclusion of multiple tumor types and the wide variety of systemic therapies included: 182 distinct drugs were included in this analysis. In addition to the risk of COVID-19 infection for patients with cancer, delays in cancer care can also negatively affect patients' cancer course. The average delay of oncologic treatment for patients in the TERAVOLT registry was 21 days.[62] Centers for Disease Control and Prevention (CDC) recommends immunocompetent individuals with COVID-19 quarantine for 10 days, while those considered immunocompromised quarantine for 20 days.[82] It remains to be seen how treatment delays will affect patient's cancer outcomes.[83]

### *1.5.4. Targeted therapies*

Treatments of NSCLC with tyrosine kinase inhibitors (TKIs) has become much more common in the 5-year overall survival of patients with lung cancer. In the initial TERAVOLT analysis of 200 patients with thoracic malignancies:[60] patients were on TKI alone. These patients were less

hospitalized (OR 0.24, 95% CI 0.077 to 0.708). Expanded analyses presented at both ASCO and ESMO, patients receiving targeted therapies do not have increased risk of death in multivariate analysis.[60-63] The degree to which this relatively favorable outcome is related to the mechanisms of these therapies as opposed to the different demographics of the patients who use them (younger, non-smokers) is not fully understood. TKIs are known to cause interstitial-like pneumonitis at varying rates and can be difficult to distinguish from COVID-19 induced pneumonia. The clinical picture of new or progressive cough, dyspnea, and/or fever can also be similar. Patients should receive COVID-19 based testing if there is either radiographic or clinical concern.[60,65,80]

### *1.5.5. Surgery*

While surgical resection strategies for lung cancer remain unvariated, many logistical challenges arise in weighing the risks and benefits to delay resection based on the severity of the COVID-19 pandemic. Limited resources including hospital beds have led to delays of non-urgent surgeries, particularly non-oncological elective interventions. Perioperative COVID-19 has been associated with mortality rates as high as 10–24% in the overall surgery population.[84,85] For patients undergoing surgery for lung cancer, the UK Lung Cancer Coalition's Clinical Advisory Group stated that increased mortality more than 40–50% has been seen in patients who contracted COVID-19 following surgery for lung cancer.[86,87] To reduce risks operation, during the incubation period for COVID-19, patients should receive COVID-19 testing prior to surgery.[65] ESMO recommends keeping all NSCLC surgeries as a priority and that surgical delays should not last more than 6–8 weeks.[86] ASCO, moreover, recommends clinicians and patients make individual determinations of risk and benefit in proceeding with surgery.[88] Finally, American College of Surgeons has published guidelines on triaging elective cases for surgical care during the COVID-19 pandemic and includes characteristics such as solid *versus* non-solid component, size of the presumed lung cancer, node positivity, and completion of induction chemotherapy.[89]

All of this information can manage triage patients treatment, as well as help thoracic surgeons for their patients in the context of COVID-19.

## 1.6 Lung Cancer Research: Impact of COVID-19 Pandemic

The COVID-19 pandemic has affected the care of patients with lung cancer and has affected multiple aspects of cancer research.[90] Since the beginning of the pandemic, there has been a decrease in clinical trial enrollment, and several active trials have been placed on hold.[91]

While data demonstrating the effect of COVID-19 specifically on lung cancer research is rare, several studies show that cancer research in general has been compromised.[92-98] The mechanisms by means of research has been compromised include decreased funding, operational concerns with tissue collection and biobanking, reduced workforce, holds on clinical trials and reduced networking opportunities for international collaborations.[47,96] In an effort to sustain cancer research during the pandemic, the National Institute of Health in the USA has implemented changes in order to increase research funding, such as including administrative supplements to existing grant awards, extending deadlines for grant applications, allowing salary support through grants, and extending eligibility periods for early-stage trainees ad fellowships.[47] As such, organizations that depend on these donations to allocate grant funds, like the American Cancer Society, the Canadian Cancer Society, and Cancer Research UK, have had to significantly reduce their budget for research funding.[95-97] Despite these budget cuts, there have been ongoing efforts to continue cancer research and determine the impact of COVID-19 on patients with lung cancer.

With the surge of COVID-19, patients requiring hospitalization have increased, leading to decreased hospital capacity and a subsequent postponement of surgeries and procedures. This has compromised tumour care and has affected the acquisition of specimens for translational and correlative studies.[91,94,99] Since patients with lung cancer are at a higher risk of infection with SARS-CoV-2 than the general population, there are concerns with the handling of specimens from patients that test positive for the virus.[99] These specimens are potentially infectious and require special procedures.[91] Finally, multiple cancer research laboratories have had to shut down, stay at home orders and to comply with social distancing strategies. This has led to the decrease of experimental resources such as patient-derived xenografts, transgenic mice, and cell lines. Other resources have been repurposed for COVID-19 research (i.e. evaluation of anticancer agents for the treatment of COVID-19). These shutdowns will lead to delays in cancer research and potentially important discoveries.[93,100-102] While the effect of the COVID-19 on lung cancer trials specifically has not been reported, cancer trials in general have been severely disrupted since the beginning of the pandemic.[47] For instance, cancer centers have generally prioritized clinical trials based on their evidence of clinical activity.[90,100,101] An analysis of data obtained from a combination of surveys, IQVIA, clinical trials.gov, and interviews revealed that only around 15–20% of trials conducted in Europe and the USA were enrolling patients at the usual rate.[97,103,104]

For investigators who continued to conduct trials, the greatest barriers to enrollment included the need for in-person patient evaluation and intravenous administration of the investigational agent. Regarding new clinical trials, investigators' main concerns included patient follow-up and type (i.e., target agents, chemotherapy, immunotherapy, or combination of these with some investigational

agents) and route of administration of the agent (oral *versus* intravenous or subcutaneous). The 60% of interviewees rated the pandemic as having a 'moderate' or 'high' impact on their trials, and 80% of respondents anticipated protocol deviations and concerns regarding missing data (suspension of, more o less, 200 clinical data).[97] Similarly, a survey of 64 investigators conducted by ASCO evaluated the pandemic's impact at a clinical trial level in both academic and community centers: a) 50% of respondents reported discontinuation of research-only laboratory collections; b) 60% reported halting screening and enrollment, and most reported that initiation visits had to be conducted remotely.[105] Investigators have had to develop innovative ways to continue to conduct cancer research, such as telemedicine for patient follow-up, as well as electronic methods for obtaining consent remotely.[97] Other strategies to mitigate direct patient contact have included the shipment of investigational agents to the patients' homes with phisician supervision via telehealth.[90] Furthermore, both US FDA and EMA published guidelines to help sponsors, investigators, and institutional review boards (IRBs) navigate clinical trials during this health crisis. These guidelines address issues such as the collection and reporting of data, different modalities for patient follow-up, and risk–benefit evaluations of continuing *versus* halting trials.[106,107]

The US FDA requested that sponsors, improved flexibility regarding protocols and authorized remote communication with patients for follow-up.[101] However, investigators have circumvented some of these difficult circumstances using different strategies that could change the way cancer research is conducted moving forward.[90]

## 1.7 WHO Classification and Genetic Tumour *Status*

The classification of tumors, in particular of lung tumors, is of fundamental importance both in order to outline a diagnostic-prognostic-therapeutic picture that is as correct, effective and reproducible as possible, and as it provides the basis for epidemiological and biological studies.

The classification of lung cancers currently in use is the one formulated by the World Health Organization (WHO) (Table 1), enjoying wide consensus among clinicians and oncologists. This classification, also in relation to the recently introduced clinical, immunohistochemical and genetic-molecular discoveries/informations that characterize tumours and which can guide clinicians in making a more informed decision regarding the setting of personalized therapy. As regards the new WHO 2015, two introductory paragraphs have been created in which the rationale for the classification of small biopsies and cytological samples is defined first, then a section of terminology and criteria for biological samples not subject to resection.[108]

This distribution system was initially proposed by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) lung andenocarcinoma classification in 2011.

The WHO classification for small biopsies also takes into account that some tumors can only be diagnosed by tumor resection analysis, so the cytological examination cannot formulate a conclusive diagnosis.[108]; therefore, molecularly targeted therapies are administered only to patients with advanced NSCLCs. An algorithm has been developed to manage biopsy samples addressing the growing need to distinguish the different histological subtypes of NSCLCs also using ancillary methods (such as immunohistochemistry) especially in advanced patients.

This section not only provides new criteria for diagnosing adenocarcinomas (ADKs) *versus* squamous cell carcinomas, which includes the use of special stains in difficult to interpret cases, but also highlights the importance of preserving the tissue for subsequent molecular studies. In the histological field, lung neoplasms can be divided into two large groups: NSCLC and SCLC. Within NSCLC we can identify three important subgroups including: SCC, ADK and large cell carcinoma (LCC). There are also other less represented groups, such as neuroendocrine carcinomas and those of the salivary type.[108]

## 1.8 Adenocarcinomas Macro and Microscopic Characteristics

***Adenocarcinoma (ADK)***

The 2015 WHO has adopted the classification recently developed by the IASLC/ATS/ERS as previousely described. ADKs classification has incorporated up-to-date advances in radiological, molecular and oncological knowledge, providing univocal diagnostic criteria and terminology.

For resection specimens, new entities have been defined such as *in situ* ADK and minimally invasive ADK to designate ADKs, mostly non-mucinous and ≤ 3 cm in size, with either pure lepidic growth or predominant lepidic growth with ≤ 5 mm invasion, respectively. For invasive ADK, the classification has introduced histological subtyping according to the predominant pattern of growth of the neoplastic cells: acinar (Image 1), papillary (Image 2), micropapillary (image 3), lepidic (formerly non-mucinous brochioloalveolar ADK) (Image 4), and solid (Image 5). Of note, micropapillary pattern is a brand new histologic subtype. In addition, four variants of invasive ADK are recognized, namely invasive mucinous (formerly mucinous brochioloalveolar ADK), colloid, fetal, and enteric. Importantly, three variants that were considered in the previous classification have been eliminated, specifically mucinous cystadenocarcinoma, signet ring cell, and clear cell

ADK. This has been introduced by the current histological classification of lung ADKs and its prognostic implication.[109]

The relative frequency of lung ADK has been increasing steadily over the past few decades, as opposed to squamous cell carcinoma, most likely as a result of spreading of low nicotine-tar cigarettes. Therefore, nowadays ADK represents by far the most frequent histologic type of lung cancer, accounting for more than 40% of the total. It slightly predominates in male patients, but not infrequently occurs in women, also relatively young, and in individuals who have never smoked. Over the last decade, the unprecedented advances in the understanding of lung ADK, with regard to radiology, molecular biology, and medical oncology, made necessary a reconsideration of its classification in view of the new knowledge, which involved not only pathologists, but also radiologists, molecular biologists, clinicians, and surgeons. As a matter of fact, the latest WHO classification is the result of an integrated multidisciplinary approach.[109]

A grading system for lung ADK has not been established. The International Association for the Study of Lung Cancer pathology panel evaluated a set of histologic criteria associated with prognosis aimed at establishing a grading system for invasive pulmonary ADK. The enrolled cases must be studied by pathologists and evaluated for the histologic parameters described subsequently. This is performed using the semiquantitative estimation of all ADKs patterns as suggested by the current WHO Classification of tumors of the lung, pleura thymus and heart 2015.[110]

*Nuclear Grade*: it was determined as follows: Grade 1: round, regular nuclei with evenly dispersed chromatin and without inconspicuous nucleoli, up to 2 to 3 the size of a lymphocyte. Grade 2: round, mildly irregular, minimally pleomorphic nuclei without inconspicuous nucleoli, up to 2 to 3 the size of a lymphocyte. Grade 3: pleomorphic nuclei with prominent nucleoli, greater than 5 the size of a lymphocyte.

*Mitotic Grade*: it was determined as follows: Grade 1: 0 to 1 mitotic figure/10 high-power field (hpf). Grade 2: 2 to 4 mitotic figures/10 hpf. Grade 3: greater than or equal to 5 mitotic figures/10 hpf. A grading system based on the predominant and high-grade patterns is practical and prognostic for invasive pulmonary ADK.[110,111]

The ADK has different epithelial markers which can be highlighted through specific immunohistochemical panels. The intensity of the marking using the immunohistochemical method (IHC) varies according to the tumor subtypes and their grading. The expression pattern of low molecular weight keratins and TTF-1 differs between mucinous and non-mucinous forms, while all ADKs are negative for CK5 / 6. More than 90% of non-mucinous forms, including the forms of BAC, are CK7 + and TTF-1 + in about 75% of these. 70% of non-mucinous forms have a CK7 + / CK20- / TTF-1 + profile.[108,109]

Some early genetic alterations in the development of ADK have been identified including the methylation of promoters, resulting in the silencing of tumor suppressor genes, and / or translocations involving oncogenes. However, it is necessary to evaluate the different histological subtypes of ADK from a genetic-molecular point of view: from the smoking or non-smoking-related forms, to those childhood cancers associated with congenital cystic adenomatoid malformation; these forms present themselves with different chromosomal aberrations. Some prognostic markers have recently been identified: immunohistochemical positivity for Cathepsin E and negativity for HSP105 (Heat Shock Protein 105 kDa), define a subgroup of ADK with a favorable prognosis; instead the expression of PCNA (Proliferating Cell Nuclear Antigen) and *Cyclin D*, from the down-regulation of *PTEN* (Phosphatase and Tensin homolog), of *NM23* (non-metastatic gene 23) and of TCL1 (T-cell Leukemia 1), negatively affect the outcome in ADK.[108,109] Pulmonary neoplasms have also been characterized through biological and molecular genetic analyzes, which have better defined the peculiarities of these cancers. *KRAS* mutation (V-Ki-Ras2 Kirsten rat sarcoma viral oncogene homolog) is frequently involved in ADK carcinogenesis, but currently, for the so-called target therapy, it is more important to establish the presence of mutations (between including gene amplifications and somatic mutations) in EGFR (Epidermal Growth Factor Receptor) gene. *EGFR* alterations, in fact, represent the major predictor of response to therapies with tyrosine kinase inhibitors.[109,112]

In particular, ADKs that develop in non-smoking patients have different clinical features from those found in smoking patients. In fact, the distinctive characteristics are supported by analyzes such as Gene Expression Profiling (GEP); ADKs with mutations involving *EGFR* gene are in fact cataloged in a single group of tumors that develop at the level of the peripheral lung parenchyma and share the same genetic expression profile. To explain the characteristics of a distinct group of lung ADKs, the concept of terminal respiratory unit has been proposed, since the tumor deriving from that specific anatomical portion possesses unique characteristics in terms of morphology, immune profile, as well as susceptibility to certain genetic alterations. Unlike the genetic alterations described in other cancers (such as sarcomas, lymphomas and leukemias), histological-molecular correlations exist within the spectrum of lung cancers.[108,112] Among these, the strongest seems to be the one documented within invasive mucinous ADK, whose origin is hypothesized from the proximal tract of the bronchial tree as a consequence of exposure to smoke, in which there is a high level of frequency of mutations affecting *KRAS* gene and not those for *EGFR*. Mutations in *EGFR* and *KRAS*, as well as rearrangements of the Anaplastic Lymphoma Kinase (*ALK*) gene, can be observed in most histological subtypes of invasive ADK. Mutations in *EGFR* gene are most often associated with the lepid and papillary type histology of non-mucinous ADK, while no mutation has been

found in micropapillary ADK. Another molecular marker, of not negligible importance, is *BRAF* (v-Raf murine sarcoma viral oncogene homolog B1), a proto-oncogene also involved in cell signaling, growth and differentiation. In the last 10 years it has a "new" molecular marker for ADK has been identified: *ALK* gene, which represents an important target for genetic-molecular investigations in order to better classify the histotype of this lung neoplasm.[108,112]

Table 1: Classification of malignant epithelial pulmunary tumours WHO 2015[108]

| | |
|---|---|
| **Squamocellular Carcinoma** | Keratinizing<br>Non- Keratinizing<br>Basalioid<br>Preinvasive lesions<br>Squamocellular Carcinoma *in situ* |
| **Adenocarcinoma** | Lepidic<br>Non mucinous<br>Mucinous<br>Acinar<br>Papillar<br>Micro papillar<br>Solid<br>Low Invasive<br>Non mucinous<br>Mucinous<br>Preinvasive Lesions<br>Atypical Iperplasia adenomatous<br>Adenocarcioma *in situ*<br>Non mucinous<br>Mucinous |
| **Adenocarcinoma Variants** | Mucinous Invasive<br>Mixed Mucinous Invasive and non-mucinous<br>Fetal<br>Colloid<br>Enteric |
| | Small Cell Carcinomas<br>Combined Small Cell Carcinomas<br>Large Cell Carcinomas<br>Combined Large Cell Carcinomas<br>Carcinoid<br>Typical<br>Atypical<br>Preinvasive Lesions<br>Lung idiophatic diffuse lung neuroendocrine with cellular hyperplasia |
| **Large cell Carcinoma** | |
| **Adenosquamous Carcinoma** | |
| **Pleomorphic Carcinoma** | |
| **Carcinoma with spindle cells** | |
| **Giant cells Carcinoma** | |
| **Carcinosarcoma** | |
| **Pulmunary Blastoma** | |
| **Unclassified Carcinoma** | Lymphoepithelial-like Carcinoma<br>Carcinoma NUT (Nuclear Protein in Testis) |
| **Salivary Gland Tumours** | Mucoepidermoid Carcinoma<br>Adenoid-cystic Carcinoma<br>Epithelial ann myoepithelial Carcinoma<br>Pleomorphic Adenoma |

## 1.9 Adenocarcinomas Genetics and Target Therapies

### *1.9.1 Main genes involved in development of ADK: EGFR, KRAS, BRAF, ALK, ROS1, HER2, PI3K, cMET and RET*

***EGFR***

EGFR is a membrane glycoprotein receptor of 170 kDa (chromosomal locus 7p11.2), expressed in ubiquitous way, composed of four functional domains: the extracellular portion, site of attachment for the ligands; a hydrophobic transmembrane region; a cytoplasmic region, containing the tyrosine kinase domain; the terminal region with tyrosine residues involved in intracellular signal transduction. EGFR belongs to the ErbB receptor family, to which four structurally related tyrosine kinase receptors belong: EGFR (ErbB-1), HER2/c-neu (ErbB- 2), HER3 (ErbB-3) and HER4 (ErbB-4). These receptors are activated by forming homodimers and/or heterodimers upon attachment of their ligands to the binding site. Mutations in *EGFR* involve a profound dysregulation of all the molecular mechanisms in which it is involved, modifying cellular homeostasis and promoting the pathogenesis of many neoplasms, including pulmonary ones. In cellular homeostasis, EGFR dimerization promotes its intracellular tyrosine kinase activity by autophosphorylation of specific tyrosine residues (Y992, Y1045, Y1068, Y1148 and Y1173) at the level of its C-terminal domain (Y = tyrosine).[113,114]

131 mutations affecting the *EGFR* gene have been identified, of which 45% involve exon 19 and 41% exon 21, followed by exon 18 (8%) and exon 20 (6%); these mutations caused amino acid substitutions. 53% were inframe deletions in exon 19 and 4% were insertions in exon 20; most of them involved the replacement of a single nucleotide. Mutations that arise in exon 20 should receive even more attention in the analytical phase, because most of these confer resistance to *EGFR* inhibitor drugs to cancer cells. Specifically, these are point mutations: the most common is represented by c.2369C>T (p.T790M) which is found in 63% of tumors treated with EGFR tyrosine kinase inhibitors as a form of acquired resistance, while primary resistance due to the p.T790M mutation is believed to be less common. In rare cases the mutation is of the germinal type; a very small number of NSCLC patients (as described in the study by Girard N. et al, 2/369 cases, 0.54%) it caused primary resistance to EGFR tyrosine kinase inhibitors.[108,113]

***KRAS***

*KRAS* gene (chromosome 12p12.1) encodes a 21 kDa protein belonging to the family of RAS proteins (superfamily of GTPases), involved in the pathways of intracellular signal transduction (including that activated by EGFR); their activation/inactivation regulates cell growth, differentiation and survival. This gene acts as a molecular on/off switch: when activated it recruits proteins necessary for the diffusion of growth factors and activates other receptors such as c-Raf (RAF proto-oncogene serine/threonine-protein kinase) and PI3-Kinase (Phosphatidylinositol 3-kinase) involved in signal translation. Recently it has been shown that mutations in this gene are involved in the development of malignant neoplasms, identifying *KRAS* as an oncogene of relevant importance.[108,115] Causes related to these mutations include an increase in cell proliferation, the ability to originate cell clones. These mutations most commonly involve exons 2 (codons 12 and 13), 3 (codons 59 and 61) and 4 (codons 117 and 146); physiologically, codons 12, 13, 59 and 61 code for a glycine, while codons 117 (lysine) and 146 code respectively for a lysine and an alanine; the simple substitution of a single nucleotide changes the amino acid frame, resulting with a production of proteins with a reduced GTPase activity.[114]

Mutations affecting the *KRAS* gene with particular reference to a subgroup of mutations (*KRAS*-G12C and -G12V) there is a worse outcome than other mutations or in the case of wild-type *KRAS*. Mutated *KRAS*-G12C and -G12V patients show an increase in signal transduction through RAL (Guanine nucleotide exchange factor with PH domain and SH3 domain-binding motif-1), decrease through AKT. This study suggests that targeted treatments and clinical trials of NSCLC patients should consider specific *KRAS* mutations. Over the years, many clinical studies have been carried out to be able to prepare a specific targeted therapy in affected patients who show mutations in *KRAS* gene. *BRAF*/*KRAS*-mutated patients treated with sorefenib had a higher DCR than patients treated with erlotinib (79% *versus* 14%); a decrease in progression free survival was associated with the group of *KRAS*-G12C and -G12V mutated cases compared to the other *KRAS*-mutated/wild-type cases. These results are similar to others obtained in similar studies, in which it is documented that in *EGFR*-mutated (and *KRAS*-wild-type) cases, there is an increase in overall survival with sorafenib when used as a third or fourth line of monotherapy.[115,116]

***BRAF***

The *BRAF* gene (chromosome 7q34) encodes a protein belonging to the RAF family with 94 KDa serine/threonine kinase activity. This protein plays a fundamental role in the regulation of the MAP/ERK signaling pathway, the effects of which are cell division, differentiation and secretion. In particular, RAF kinases belong to a family composed of three serine/threonine kinases: RAF1,

BRAF and ARAF, which are part of the RAS-MAPK signal transduction cascade and are able to phosphorylate MEK. As growth factor stimulation occurs, RAF1 (or c-RAF) is activated by the GTP-RAS binding and recruited by the cell membrane. This activation process is tightly regulated by a number of factors including both phosphatases (i.e. PP1, PP2A), kinases (i.e. ERK, AKT, PKC), and proteins that directly bind RAF1 (i.e. RKIP, KSR, HSP90); it is believed that the latter is able to dimerize with wild type forms of BRAF in the RAS-dependent process. Mutations in *BRAF* gene may be found in cancer; in particular, the most commonly encountered point mutation is c.1799T>A (p.V600E) (exon 15) at the level of the kinase domain. Although the wild type form of BRAF and RAF1 is strongly activated by the growth factor signal via the RAS and SRC pathway, while ARAF is modestly activated. All three RAF isoforms are considered to have a potential oncogenetic role. Germline mutations in this gene lead to heart failure or mental retardation with typical facies; while somatic mutations are present in different types of cancer, including malignant melanoma, colorectal, thyroid and lung carcinoma.[108,114,117]

All mutations were found in the kinase domain through a single amino acid substitution (p.V600X). BRAF is a serine/threonine kinase protein commonly activated by a point mutation in cancer, which could provide new therapeutic opportunities for the treatment of malignant melanoma. Suspected somatic mutations in *BRAF* gene have been studied and observed in at least 43 different cancer cell lines including melanoma (59%), colorectal cancer (18%), lung (3%), cancer of the ovary (4%), breast (2%), liver (14%), gliomas (11%) and finally sarcomas (9%).

Regarding personalized therapies in the area of lung cancer (NSCLCs), mutations in *BRAF* gene were most commonly found in 2.8% of ADKs with micropapillary histotype in non-smoking female patients, accounting for 58% of all. Mutations of *BRAF* gene documented in general in neoplasms. This mutation is not commonly associated with the long-term survival of patients who present it, in fact in these cases the disease-free survival is shorter, compared to wild-type cases. In patients with NSCLCs, the therapeutic response (with *BRAF*-mutant) to vemurafenib or dabrafenib may be complete or partial, although not lasting. The use of sorafenib has also given good results in patients with NSCLCs who presented with *BRAF*-mutated.[117]

The clinical study presented by the “European Lung Cancer Conference” published encouraging data, describing clinical cases that responded well to therapy even for long periods. Most patients with non-V600E mutations did not respond to *BRAF* inhibitors and had a significantly worse outcome. The mechanism of primary resistance to *BRAF* inhibitors that establish non-V600E mutations in cases of NSCLCs is not fully understood. This could be explained by the tertiary structure of the protein: for example, three-dimensional models of mutated *BRAF* protein at codon

469 (p.G469L) were constructed that demonstrated the steric modification of the protein that compromises the binding of the Vemurafenib and Dabrafenib molecules.[108,117]

***ALK***

ALK is a receptor with a tyrosine kinase activity of 200 kDa, belonging to the superfamily of insulin receptors, encoded by *ALK* gene located on chromosome 2 (2p23). ALK receptor belongs to the RPTK (Receptor protein-tyrosin kinase) family. These receptors are composed of three domains; one extracellular, attachment site for the ligand, one transmembrane portion and one cytoplasmic portion containing the catalytic site. The attachment of the ligand to the extracellular site induces the activation of kinases on the cytoplasmic side of the receptor, activating a pathway of transduction of the intracellular signal. The activated RPTKs are both self-phosphorylating and capable of phosphorylating cytoplasmic substrates, leading to the activation of signaling cascade; finally, they are able to modulate gene expression. Furthermore, RPTKs play a very important role in cell proliferation and differentiation. When RPTKs possess constitutive kinase activity (caused by point mutations, amplifications or rearrangements of the corresponding genes), they have an oncogenic potential. When ALK is not activated by its ligand, the inactive tyrosine kinase site is cleaved by the caspases and promotes apoptosis; when ALK is activated by its ligand, it inhibits the apoptotic process itself.[114]

What still remains controversial is the very nature of the ALK ligand. In some studies, two heparin-binding growth factors, PNM (Pleiotropin) and NEGF2 (Neurite Growth-promoting Factor 2), have been proposed as possible ligands, having pleiotropic activity and involved in normal development and tumor growth. For the other members of the RPTK family, ALK also has an oncogenic potential in the specific case of a fusion event with another partner gene. The first data confirming this theory were observed through the study of anaplastic large cell lymphomas (ALCL), in which the presence of the translocation t(2;5)(p23;q35) was recurrent, which generates the chimeric oncogene *NPM-ALK* (NPM: nucleophosmin), with a frequency of 85%. The amino acid sequence of NPM-ALK shows that in the gene product the kinase domain of ALK is bound to that of NPM (ubiquitously expressed non-ribosomal nucleolar phosphoprotein).[108,118]

*ALK* gene is involved in translocations with different partner genes including: *NPM*, *MSN* (moesin), *TPM3* (tropomyosin-3), *ATIC* (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase / *IMP* cyclohydrolase gene previously mapped to [2q34 -q35]), *TFG* (transforming growth factor), *CARS* (cysteinyl-tRNA synthetase), *CLTC* (clathrin) and *KIF5B* (kinesin-1 heavy chain). Positivity for translocations involving *ALK* gene is strongly associated with acinar (including cribriform

morphology) and solid ADK subtypes, particularly in the presence of signet ring cells. Also for the aberrations that characterize this gene, as for *EGFR*, a correlation to tobacco consumption has not been documented, but, on the contrary, specific etin-associated aberrations have not been described.[118]

***ROS1***

ROS1 is a receptor with tyrosine kinase activity of 259 kDa, belonging to the Sevenless tyrosine kinase subfamily of insulin receptors, encoded by *ROS1* gene (V-Ros avian UR2 Sarcoma virus Oncogene homolog 1), located on chromosome 6 (6q22.1). Coding for a type I integral membrane protein, with tyrosine kinase activity; the ROS1 receptor therefore belongs to the RPTK family (Receptor Protein-Tyrosin Kinase). The protein is highly expressed in different type and subtype of cancer cell lines that can act as both a receptor and a growth and/or differentiation factor.

Translocations involving this gene account for approximately 1.3% of total ADKs, which are characterized by lymph node metastases and a histological pattern of solid or papillary tumor growth. The first recognized fusion protein was discovered in glioblastoma and was named *FIG* (Fused In Glioblastoma).[114] FIG-ROS1 protein is not the result of a real translocation, but of an interstitial deletion of 240Kb at the level of chromosome 6q21 (del6q21q21), which makes tyrosine kinase activity constitutively active of the receptor. In addition to the FIG-ROS1 isoform found in glioblastoma, other short and long types have been identified as potential drivers of cholangiocarcinoma tumorigenesis. Proteomics studies have examined the tyrosine kinase activity of ROS1 through the phosphorylation of its receptor domain, discovering that in addition to FIG, it has two other partners involved in the tumorigenesis of NSCLCs: CD74 and SLC34A2 (which encodes a Solute carrier protein). Translocations involving ROS1-FIG / -CD74 / -SLC34A2 arise from the same 6q21 gene *locus*.[108,118]

***HER2***

*HER2* encodes a 185 KDa membrane glycoprotein, belonging to the family of epidermal growth factor (EGF) tyrosine kinase receptors, chromosomal *locus* 17q12. It is able to activate downstream PI3K-AKT and MEK-ERK signaling pathways, without having been described any specific ligand for this receptor, which is activated thanks to homo/heterodimerization with other members of the ErbB family. The amplification and/or over-expression of *HER2* gene has been described in various types of cancer including ovarian and breast cancer and glioblastoma. EGFR receptor, as previously described, belongs to the ErbB family, which is identified thanks to its four members: EGFR (ErbB-1 or HER1), ErbB-2 (HER2), ErbB-3 (HER3), finally ErbB-4 (HER4).[119]

Mutations in the *HER2* gene were found in about 2-4% of patients with NSCLC, being the most representative driver of neoplastic proliferation compared to other genes belonging to the same family.[108,114,119]

The most commonly encountered mutations are inframe insertions in exon 20 (such as: p.G776_777insVC), which constitutively activate the transduction pathway of the AKT and MEK signal. HER2 protein over-expression and gene amplification can be present respectively from 6 to 35% and from 10 to 20%. A selected population of NSCLC patients negative for mutations involving *EGFR, KRAS* and *ALK* genes, may have mutations in *HER2* exon 20 with a frequency of up to 6% (histotype adenocarcinoma). Unlike other biomarkers involved in the development of lung cancer, *HER2* still remains poorly described and researched, although the response to target therapies (biological drugs such as trastuzumab, lapatinib) in breast cancer is already well known.[108,114,119]

***PI3K***

PI3K (phosphoinositide 3-kinase or phosphatidylinositol-3-kinase or PI 3-kinase), chromosomal *locus* 7q22.3, encodes a family of enzymes involved in complex cellular mechanisms such as growth, proliferation, differentiation, motility and intracellular survival; mechanisms also involved in the development of tumor pathologies. This class of enzymes is also known as phosphatidylinositol-3-kinase. The metabolic pathway, with the oncogene Phosphatidylinositol-4,5-Biophosphate 3-Kinase Catalytic Subunit α, *PIK3CA* and with the tumor suppressor gene *PTEN*, is involved in the phenomena of insensitivity of tumors to insulin and the Insulin Growth Factor 1 receptor (IGF1), in subjects subjected to caloric restriction.

PI3K is composed of a regulatory subunit of 85kDa (Class IA of the isoforms of PI3K with regulatory subunits p50α, p55α, p55γ, p85α and p85β) and a catalytic subunit of 110kDa (Class IA of PI3K consisting of the catalytic subunit isoforms p110α, p110β) and p110δ; Class IB consisting of the γ isoform). PI3K p110 type α and β, are ubiquitously expressed in association with leukocytes. It has recently been discovered that mutations activating the α-subunit may play a role in the development of some forms of cancer. The protein encoded by *PI3KCA* gene represents the catalytic subunit, which uses adenosine tri-phosphate (ATP) to phosphorylate the hydroxyl in position 3 present on the inositol ring of phosphatidylinositol [PtdIns, PtdIns4P and PtdIns (4,5) P2].[114] In particular, these mutations are generally present in the catalytic domain and have been identified in about 1-3% of NSCLCs, more commonly found in SCCs than in ADKs. Unlike other driver-type mutations, *PI3KCA* mutations may be present in association with other genetic alterations (such as those affecting *EGFR* and/or *KRAS* genes), suggesting a marginal role in the

development of the neoplasm. However, in vitro studies carried out on lung cancer cell lines characterized by *PI3KCA* mutations or Copy Number Gain (CNG) aberrations showed increased PI3 kinase activity sensitive to pharmacological inhibition with small molecules. In vivo studies on mouse models, on the other hand, have shown the oncogenic activity of *PI3KC*A, whose mutations have allowed the development of ADKs. It has also been documented that in NSCLCs it is also possible to find a phenomenon of genetic amplification of *PI3KCA* gene, especially in SCCs, in about 5% of the cell lines studied. Point mutations in the AKT gene are equally rare (0.5-2% of NSCLCs, especially in SCCs), constitutively activating the PI3K / AKT / mTOR pathway.[108,120]

***cMET***

*cMET* (MET Proto-Oncogene, Receptor Tyrosine Kinase) encodes a member of the receptor tyrosine kinase family of proteins and the product of proto-oncogene c*MET*. The encoded preproprotein is proteolytically processed to generate alpha and beta subunits that are linked via disulfide bonds to form the mature receptor. Further processing of the beta subunit results in the formation of the M10 peptide, which has been shown to reduce lung fibrosis. Binding of its ligand, hepatocyte growth factor, induces dimerization and activation of the receptor, which plays a role in cellular survival, embryogenesis, and cellular migration and invasion. Mutations in *cMET* gene are associated with papillary renal cell carcinoma, hepatocellular carcinoma, and various head and neck cancers. Amplification and overexpression of this gene are also associated with multiple human cancers. *cMET* alterations are drivers of human cancer. Amplification and overexpression have been reported in several cancers, and make the receptor's activity independent of HGF.[114,121]

Gene fusions also decouple kinase activity from the cell membrane and render it constitutively active. Finally, exclusion of the juxtamembrane (JM) domain of the kinase by skipping of exon 14 activates the kinase.[108,114] Mutations leading to *cMET* exon 14 skipping are the most commonly reported oncogenic *cMET* mutations, and as with many other oncogenic drivers, coexistence of *cMET* exon 14 with other oncogenic drivers is rare. Mutations may be associated with *cMET* amplification, with a co-occurrence rate between 0% and 40.5%. *cMET* amplification is caused by an increase in the copy number of *cMET* gene and has been identified as a resistance mechanism with *EGFR* mutation–positive NSCLC. Main agents are being investigated in patients with NSCLC with either *de novo cMET* amplification or presence of *EGFR* mutation disease.[108,121] Importantly, both *cMET* exon 14 and/or amplifications are associated with poor prognosis in patients with NSCLCs. *cMET* exon 14 cancers, the proper transcription process of *cMET* gene is disrupted by underlying alterations in the intronic regions surrounding exon 14, alterations within exon 14 itself, or complete genomic deletion of exon 14. Point mutations within exon 14, such as

Y1003X or D1010X, may also mimic the loss of this region. Mechanisms behind the oncogenesis are incompletely explored, and multiple mechanisms may be involved; however, it is believed that loss of this region results in impairment of proper receptor degradation, leading to overactive *cMET*-mediated signaling and thus cell proliferation and tumor growth.[108,114,121]

***RET***

This gene encodes a transmembrane receptor and member of the tyrosine protein kinase family of proteins. Binding of ligands such as GDNF (glial cell-line derived neurotrophic factor) and other related proteins to the encoded receptor stimulates receptor dimerization and activation of downstream signaling pathways that play a role in cell differentiation, growth, migration and survival. The encoded receptor is important in development of the nervous system, and the development of organs and tissues derived from the neural crest. This protoncogene can undergo oncogenic activation through both cytogenetic rearrangement and activating point mutations. Mutations in this gene are associated with Hirschsprung disease and central hypoventilation syndrome and have been identified in patients with renal agenesis. *RET* mutations and the *RET* fusion *RET-PTC* lead to activation of this tyrosine kinase receptor and are associated with thyroid cancers. *RET* point mutations are the most common mutations identified in medullary thyroid cancer (MTC) with germline and somatic mutations in *RET* associated with hereditary and sporadic forms, respectively. The most common somatic form mutation is M918T (exon 16) and a variety of other mutations effecting exons 10, 11 and 15 have been described. The prognostic significance of these mutations have been hotly debated in the field, however, data suggests that some *RET* mutation may confer drug resistence. No *RET*-specific agents are currently clinically available but several promiscuous kinase inhibitors that target *RET*, among others, have been approved for MTC treatment.[108,114,118]

In NSCLC, chromosomal rearrangements between *RET* gene and another domain, most commonly kinesin family 5B (*KIF5B*) and coiled coil domain containing-6 (*CCDC6*), lead to overexpression of RET protein. *RET* fusion occurs in 1–2% of NSCLCs, particularly in younger, non-smoking patients with adenocarcinoma histology, and they appear to be associated with a high risk of metastasis to the brain. In contrast, *KIF5B-RET* and *CCDC6-RET* fusion genes have been identified in 70 to 90% and 10 to 25% of tumors, respectively. *RET* fusion are thought to be exclusive of *EGFR*, *ALK*, *KRAS* and *BRAF* mutations, suggesting that it has its own oncogenic driver potential. A number of *RET* fusion inhibitors have recently been approved, while others are in clinical trials. Patients with *RET* fusions have minimal response to immunotherapy.[108,114,118]

### *1.9.2 Adenocarcinomas First and Second-Generation Therapies*

Precision oncology is now the evidence-based standard of care for the management of many advanced NSCLCs. Expert consensus has defined minimum requirements for routine testing and identification of *EGFR* mutations (15% of tumors harbor *EGFR* exon 19 deletions or exon 21 p.L858R substitutions) and *ALK* rearrangements (3-5% of tumors) in advanced ADKs. Application of palliative targeted therapies with oral TKIs in advanced/metastatic ADKs harboring abnormalities in *EGFR* (gefitinib, erlotinib, afatinib) and *ALK/ROS1/cMET* (crizotinib) has consistently led to more favorable outcomes compared with traditional cytotoxic agents. In addition, mutations leading to resistance to first-line *EGFR* and *ALK* TKIs can now be successfully inhibited by approved third-generation *EGFR* TKIs (osimertinib, rociletinib) and second-generation *ALK* TKIs (ceritinib, alectinib). Notably, increasing feasibility, accessibility, and application of molecular profiling technologies has permitted dynamic growth in the identification of actionable driver oncogenes. Emerging genomic aberrations for which TKIs have shown impressive results in clinical trials and expansion of drug labels for approved agents are awaited include *ROS1* rearrangements (1–2% of tumors, drug: crizotinib) and *BRAF*-V600E mutations (1–3% of tumors, drugs: vemurafenib, dafrafenib combined with trametinib). Evolving genomic events in which TKI responses have been reported in smaller series include *cMET* exon 14 skipping mutations (2–4% of tumors, drug: crizotinib); high-level *cMET* amplification (1–2% of tumors, drug: crizotinib); *RET* rearrangements (1% of tumors, drug: cabozantinib); and *ERBB2* mutations (2–3% of tumors, drug: afatinib), among others. Unfortunately, the most common genomic event in NSCLS (including ADKs), *KRAS* mutations (25–30% of tumors), is up to date targetable with in development small molecule inhibitors.[116] Here, it is currently approved, emerging, and evolving systemic precision therapies matched with their driver oncogenes for the management of advanced NSCLC.[122]

More in details, new personalized therapies have been introduced in the last decay, aimed at the treatment of NSCLCs subtypes, especially ADKs, characterized by specific mutations.

First mutations to be identified, studied and exploited for therapeutic purposes were those in *EGFR* gene: these mutations predict sensitivity to first and second-generation *EGFR* TKIs such as Erlotinib, Afatinib, Gefinitnib and Icotinib. Response rate and progression-free survival (PFS) with *EGFR* TKIs are superior to standard first-line platinum doublet chemotherapy, making them the standard of care.[123] However tumours variability develop acquired resistance in 9-13 months after treatment initiation. Several mechanism of acquired resistance have been reported, bypass track signalling pathways and histologic transformation. *EGFR* exon 20 amino acid subtitution in position 790 (threonine to methionine, p.T790M), reduces first-generation *EGFR* TKIs binding, and

accounts for over half of acquired resistence mechanism, enhancing the ATP-binding affintity to the EGFR-mutant receptor kinase domain.[124,125] Knowlwedge of acquired resistance mechanism to *EGFR* TKIs was one of the triggers behind the development of the so-called target therapies, with the introduction of third-generation *EGFR* TKIs, resistant to p.T790M *EGFR* mutations, such as Osimertinib (AZD9291).

Osimertinib is the third-generation *EGFR* TKI to recive EMA and FDA approval for metastatic *EGFR*-mutant NSCLC patient with acquired *EGFR* p.T790M mutation tested in tumor tissue and/or liquid biopsy.[123,126]

There were no therapies for targeting *KRAS* mutation lung cancer approved. *KRAS* mutation are present in up to 30% of NSCLCs, especially in ADK histotype; *KRAS* molecular characteristics and marked affinity to a specific substrate have led to define this gene as un undruggable alteration. Despite that, many attemps have been made to develop drugs capable to targeting *KRAS* signaling, but, until a few years ago, these efforts have been unsuccessful.[127]

*KRAS* p.G12C mutation occurs in 13% of NSCLC; the amino acid substitution in *KRAS* exon 2, position 12 (form glycine to cysteine), favors the active KRAS protein form, resulting in redominatly GTP-bound KRAS oncoprotein and enhanced proliferation and survival in toumor cells. Cysteine mutated resides newt to a P2 pocket, in which is present only the inactive conformation of GDP-bound KRAS: this site has been exploited to estabilish covalent inhibitors of KRAS. Sotorasib (AMG 510) is a small molecule that specifically and irreversibly inibhits *KRAS* p.G12C mutation throught a unique interaction with P2 pocket. This drug is able to inhibit nearly all detectable phosphorylation of Extracellular Signal-regulated Kinase (ERK), a key downsteram effector of KRAS, leading to durable and complete tumor suppression.[116]

However, there is a subgroup of NSCLCs cases that express *EGFR* gene in wild type form: these patients do not respond to therapy; however, more in-depth genetic-molecular studies have described that these patients carry novel chromosomal rearrangements that confer resistance to *EGFR* inhibitor therapy.[108]

Crizotinib, the first-generation *ALK* inhibitor, has been succeded by a second-generation therapies, Alectinib and Brigatinib, that have demostrated superior efficacy and better central nervous system (CNS) activity compared to Crizotinib.

Second-generation therapies *ALK* inhibitor has been shown to be superior to chemotherapy in *ALK*-rearranged disease with good CNS activity. Initial response to *ALK* inhibitors is not always durable; resistance mechanisms can occur as on-target or off-target alterations. Lorlatinib is a third-generation *ALK* inhibitors, that demontrated activity in the treatment naïve setting and resistance to crizotinib and second-generation *ALK* inhibitors. Lorlatinib ha salso shown to improve PFS in

patientins harboring ALK-EML4 variant isoform 3, associated with the development of *ALK* resistance mutation such as p.G1202R (glycine to lysine substitution in position 1202).

This poin resistance mutation has been reported to occur in 21% of patients treated with Ceritinib, 29% of patients on Alectinib, 43% of patients on Brigatinib. Ceritinib is a III phase drug like Crizotinib. Ensartinib, a new first-line *ALK* inhibitor, has demostraded efficacy in Alectinib refractory disease.[128]

*RET*-fusion were first identified in 2012. At that time, only multikinase inhibitors with some degree of preclinical anti-*RET* activity such as Carbozatinib and Vandetinib, were avaiable in the clinic. Unfotunately, this agents were associated with limited response and durability in *RET*-fusion-positive NSCLC, probably because of poor pharmacokinetic features and substantial side effects resulting from non- *RET* kinase inhibition with a median PFS of only 6 months in 73% of treated patients. In 2017 begun the clinical testing of Selpercatinib, presenting the opportunity to explore the efficacy and safety of a selective *RET*-inhibitor in this genetically defined lung-cancer subgroup.[129] Selpercatinib had rapid and durable antitumor activity in patients with advanced *RET*-fusion-positive lung cancer, with a better outcome, efficacy and safety effects compared to those previousely achived with multikinase inhibitors.[130,131]

Patients had meaningful benefit: Selpercatinib activity seens to be broadly similar to targeted therapy in patients affected of NSCLCs that harbours other estabilisher oncogenic drivers, such as *EGFR* mutations, *ALK* and *ROS1* fusions.[129]

## 1.9 Humanitas Research Hospital Screening Program

The pandemic has changed the way clinical research is being conducted. Suspension of clinical activities has affected the ability to recruit patients, comply with protocol-mandated visits, and has affected the acquisition of laboratory and radiological data that are essential for both trial endpoints and correlative studies. It is necessary to reporte reaching out to the ethic committees regarding concerns around staff safety (30%) and protocol deviations (27%). The pandemic has affected several aspects of cancer research, from preclinical studies to clinical trials. These are likely to decrease cancer discovery both in the short and long term.

Given these premises, it was inevitable for our Institute, which is present one of the most important Cancer Centers of the north of Italy in Milan, not to establish a protocol that envisaged the constant protection and care of cancer patients.

During the pandemic, at least 8546 cases affected by COVID-19 arrived at the attention of Humanitas Clinical Institute. These cases have been evaluated by means of triage procedures, clinically characterized, and assessed by CT scan.

In about 38% of cases, it was not only highlighted the clinical virus signs, but, moreover, patients also showed presence of solid tumors, often attributable to the diagnosis of lung or breast disorders even in the early stages. If the patient's virological clinical situation allowed, these patients were also monitored for neoplasm. Between March 2020 and December 2021, 2017 cases of ADKs were isolated and analyzed, of which, 481 patients (23.8%) presented an early cancer stage, while the remaining part presented stage II or III-IV; a fraction of patients (30%) have received a ADKs diagnosis, coming to the attention of our center not because they were affected by COVID-19, but because they were included in the standard monitoring and diagnosing cancer process.

Hence the need to create an internal network within our Institute, strengthened by several professionals: Rediologists and Sonographers, Thoracic Surgeons, Oncologists, Pathologists and Molecular Biologists. A multidisciplinary team that joins forces to give the patient the best possible cares in pandemic situation.

# 2. AIM OF WORK

Over the past two years, the pandemic has created an emergency, which had to be solved by looking for a different way to make cancer patients access hospital care in a relatively short period of time. The organization and management of cancer patients, as previously mentioned, has had to change: many patients have been identified who had tumors in the chest in the early stages (by means of TC scan triage procedure). It is for this reason that a multidisciplinary team was established to monitor patients with cancer, especially ADKs, mostly affected by COVID-19.

Objective of this study, organized by this multidisciplinary group born during the pandemic, is to enroll patients with cancer; at this juncture we will focus more on lung pathology, characterizing each case, from histological and molecular point of view, trying to pay more attention to patients with ADKs at early stages, trying to identify the presence of driver mutations that can impact from the therapeutic cares and to prevent possible relapses and progression of the pathology.

Setting up a screening in early ADKs stage, allow us to monitor the disease from the earliest tumour events and try to avoid the development of the disease that inevitably has a poor prognosis.

Patients, starting from March 2020 were enrolled by CT scan and triage procedures at time zero, biopsied and\or underwent surgical resection, evaluated from a histological point of view, finally characterized from a molecular *status* by means of:

- Extraction of nucleic acids from formalin-fixed and paraffin-embedded tissue samples (FFPE);
- Evaluation of the mutational *status* of the main driving genes of ADKs by Sequenom MassARRAY® [Mass Spectrometry (MS)]: *EGFR, KRAS, NRAS, BRAF, PIK3Ca* and *ALK*;
- Evaluation by means of Real Time-PCR of the fusion transcripts of the genes: *ALK, ROS1, cMET* and *RET* [alternatively with methods such as immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) in case of rare starting material];
- Real Time-PCR monitoring of primitive mutations sensitive to first-generation TKIs of *EGFR* gene and detect the possible presence of the resistance point mutation, i.e., p.T790M in cases of disease progression starting from very small re-biopsy or liquid biopsy;
- Sanger Sequencing for studying *cMET* exon 14 skipping mutations in some selected cases.

From May 2021 the Ion Torrent™ Genexus™ Integrated Sequencer System (NGS) method has been introduced: for the last year the goal was to perform a retrospective re-evaluation of cases tested negative with Sequenom MassARRAY® and analyze new enrolled cases of ADKs.

All results obtained came from a comparison and intralaboratory validation study of methods used to define the molecular and genetic *status* of lung tumors; at the same time correlate them with the clinical and prognostic data for each patient, as well as interface results obtained with data relating to follow-up and clinical outcome of affected patients with cancer and COVID-19 infection. All clinical data about terapeutic, prognostic and outcome of patient, as well as the immunological *status* (related to the vaccine), will be collected and better discussed at the end of the study estabilished by June 2022 and discussed in a separated clinical study.

# 3. MATERIAL AND METHODS

## 3.1 Enrolled Patients

Starting from March 2020 to December 2021, 8546 cases affected by COVID-19 or suffering from seasonal flu (easily confused with COVID-19 and discriminated by nasopharyngeal molecular swab), arrived at the attention of Humanitas Clinical Institute. These cases have been evaluated by means of triage procedures, clinically characterized, and assessed by CT scan. If patient's virological clinical situation allowed, it was possible try to take care of neoplasm. Between March 2020 and February-March 2021, 1447 cases of ADKs were isolated and selected in a sub-cohort of patients, analyzed by means of molecular and genetic methods. The 23.3% presented an early stage ADKs, while the remaining part were classified stage II or III-IV. Starting from February-March, until December 2021, 570 new cases were enrolled using the same selection criteria for the study. Of these, 26% were early stage (148/570 cases). The pandemic made it necessary to perform a huge number of thoracic CT-scans, which allowed the detection of an equally high number of tumors in the thoracic area, especially early-stage type.

## 3.2 Samples

All enrolled patients underwent CT-guided tissue biopsy for the first diagnosis. Of these 481, not all were able to undergo surgical resection, however, as these were early tumor stages, 94% were able to benefit from surgery, as concern protocols for stage I-II lesions. 1536 Patients in advanced stages III-IV were analyzed from a genetic and molecular *status*, starting exclusively from biopsy tissue samples, while for the other cases it was preferable to wait for the surgery tissue. We collected 453/481 surgery samples (form stages I-II) and 2473 biopsies from both early and advanced stages. Many patients could not benefit from surgical, especially with the diagnosis of advanced lung ADK stages III and IV); for this reason, they underwent multiple biopsy samples, where possible. Total surgery samples collected even from advanced stages was 776/2017 (38.4%).

This condition depends a lot on the tumor position; is not always easy to reach the specific anatomical chest part that could be particularly favorable to bleeding/embolism, so the taken biological material, is not always in an optimal quantity and quality for subsequent investigations. All tissue samples were processed following the laboratory standards for pathological anatomies; at time of the molecular investigations, the starting material, except for liquid biopsies, was exclusively FFPE.

Of all cases under study, we evaluated in parallel, in predefined timings and in accordance with the state of health of each patient, the tumor molecular *status* by means of liquid biopsy (total collected liquid biopsies was 1516).

## 3.3 Nucleic Acid Extraction

### *3.3.1 DNA Extraction*

Starting from FFPE biopsies and surgery samples, a semi-automatic Promega Maxwell RSC (Promega, Italy) instruments has been used, following manufacturer's instructions, with Maxwell® RSC DNA FFPE Kit (Promega, Italy), allowing genomic DNA purification from FFPE tissue samples. DNAs were eluted in 50µl of DNase Free Water (supplied by Kit) and quantified with Nanodrop Spectrometer (ThermoFisher Scientific, Italy, Monza).

### *3.3.2 RNA Extraction*

Starting from the same FFPE biopsies (with enough material) and surgery samples, a semi-automatic Promega Maxwell RSC (Promega, Italy) instruments has been used, following manufacturer's instructions with Maxwell® RSC RNA FFPE Kit (Promega, Italy), allowing RNA purification from FFPE tissue samples. RNAs where eluted in 50µl of DNase Free Water (supplied by Kit) and quantified with Nanodrop Spectrometer (ThermoFisher Scientific, Italy, Monza).

### *3.3.2 ctDNA Extraction*

Starting from whole blood collected in Vacutainer with EDTA or Sodium Citrate anticoagulants:

- Blood samples must be processed as soon as possible with a serum centrifuge 2000rpm for 20' and collect the plasma in a 1.5ml vial.
- Extract ctDNA, starting directly from 1.5ml of plasma with semi-automatic Promega Maxwell RSC (Promega, Italy) instruments, following manufacturer's instructions with Maxwell® RSC cctDNA Kit (Promega, Italy), automated DNA isolation. DNAs where eluted at least in 50-60µl of Elution Buffer (supplied by Kit), and quantified with Nanodrop Spectrometer (ThermoFisher Scientific, Italy, Monza).

## 3.4 SEQUENOM® MassARRAY®

Patients DNAs were analyzed by means of Matrix-Assisted Laser Desorption\Ionization Time-of-Flight Mass Spectrometry Technology (MALDI-TOF), using Sequenom instrument (Diatechpharmacogenetics, Jesi, Italy). This method consists of an initial *locus* specific PCR reaction, followed by single base extinction using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. Using this method, the distinct mass of the extended primer identifies the SNP allele. A commercial CE-IVD kit specific to detect NSCLCs main hot-spot mutations has been used: Myriapod®Lung Status (Diatechpharmacogenetics, Jesi, Italy), that can detect 307 SNPs of *EGFR, KRAS, BRAF, PIK3CA, NRAS, ALK, ERBB2, DDR2, MAP2K1* and *RET* genes with 5% of sensitivity. The experiments were performed following manufacturer's instructions, starting from at least 20ng/µl of DNA for each single reaction. Results were analyzed by means of Typer 4.0 software.(Figure 1)

*Figure 1: MS hot-spot mutation assays*

| Saggio | Allele | Mutazione |
|---|---|---|
| L01 | AF | WT |
| L01 | AF2 | NRAS G12V |
| L01 | AF3 | NRAS G12A |
| L01 | AF4 | NRAS G12D |
| L02 | AF | EGFR L861P |
| L02 | AF2 | EGFR L861Q |
| L02 | AF3 | EGFR L861R |
| L02 | AF4 | WT |
| L03 | AF | \ |
| L03 | AF2 | WT |
| L03 | AF3 | EGFR D770_N771insG |
| L03 | AF4 | EGFR ins |
| L04 | AF | EGFR L747_T751delLREAT |
| L04 | AF2 | EGFR L747* |
| L04 | AF3 | EGFR L747* |
| L04 | AF4 | WT |
| L05 | AF | ALK G1269A |
| L05 | AF2 | ALK G1269E |
| L05 | AF3 | WT |
| L05 | AF4 | ALK G1269V |
| L06 | AF | EGFR ins |
| L06 | AF2 | EGFR ins |
| L06 | AF3 | WT |
| L06 | AF4 | EGFR H773_V774insH |
| L07 | AF | KRAS G12R |
| L07 | AF2 | KRAS G12S |
| L07 | AF3 | WT |
| L07 | AF4 | KRAS G12C |
| L08 | AF | EGFR E709E |
| L08 | AF2 | EGFR E709D |
| L08 | AF3 | EGFR E709D |
| L08 | AF4 | WT |
| L09 | AF | EGFR T751_I759>REA |
| L09 | AF2 | EGFR del |
| L09 | AF3 | WT |
| L09 | AF4 | EGFR S752_I759delSPKANKEI |
| L10 | AF | EGFR L833L |
| L10 | AF2 | EGFR L833M |
| L10 | AF3 | EGFR L833V |
| L10 | AF4 | WT |
| L11 | AF | EGFR E746_T751>A |
| L11 | AF2 | WT |
| L11 | AF3 | EGFR E746G |
| L11 | AF4 | EGFR E746_S752>V |
| L12 | AF | WT |
| L12 | AF2 | EGFR D770fs*61 |
| L12 | AF3 | EGFR V769_D770insERG |
| L12 | AF4 | \ |
| L13 | AF | EGFR P848R |
| L13 | AF2 | EGFR P848L |
| L13 | AF3 | WT |
| L13 | AF4 | EGFR P848Q |
| L14 | AF | EGFR H773_V774insAH |
| L14 | AF2 | \ |
| L14 | AF3 | WT |
| L14 | AF4 | \ |
| L15 | AF | PIK3CA H1047R |
| L15 | AF2 | PIK3CA H1047L |
| L15 | AF3 | PIK3CA H1047P |
| L15 | AF4 | WT |
| L16 | AF | EGFR E709Q |
| L16 | AF2 | EGFR E709K |
| L16 | AF3 | WT |
| L16 | AF4 | EGFR E709fs*1 |
| L17 | AF | WT |
| L17 | AF2 | KRAS G12V |
| L17 | AF3 | KRAS G12A |
| L17 | AF4 | KRAS G12D |
| L18 | AF | WT |
| L18 | AF2 | BRAF L597I |
| L18 | AF3 | BRAF L597V |
| L18 | AF4 | BRAF L597S |
| L19 | AF | \ |
| L19 | AF2 | EGFR K745_A750delKELREA |
| L19 | AF3 | WT |
| L19 | AF4 | EGFR E746_A750delELREA (2235_2249del) |
| L20 | AF | DDR2 I638V |
| L20 | AF2 | DDR2 I638F |
| L20 | AF3 | DDR2 I638L |
| L20 | AF4 | WT |
| L21 | AF | EGFR del |
| L21 | AF2 | EGFR E746_A750delELREA (2236_2250del) |
| L21 | AF3 | WT |
| L21 | AF4 | EGFR E746_T751delELREAT |
| L22 | AF | \ |
| L22 | AF2 | \ |
| L22 | AF3 | EGFR K745_E749delKELRE |
| L22 | AF4 | WT |
| L23 | AF | PIK3CA E545Q |
| L23 | AF2 | PIK3CA E545K |
| L23 | AF3 | WT |
| L23 | AF4 | PIK3CA E545* |
| L24 | AF | EGFR D770fs*59 |
| L24 | AF2 | EGFR ins |
| L24 | AF3 | WT |
| L24 | AF4 | EGFR V769_D770insCV |
| L25 | AF | KRAS Q61Q |
| L25 | AF2 | KRAS Q61H |
| L25 | AF3 | KRAS Q61H |
| L25 | AF4 | WT |
| L26 | AF | WT |
| L26 | AF2 | EGFR L858M |
| L26 | AF3 | EGFR L858V |
| L26 | AF4 | EGFR L858L |
| L27 | AF | WT |
| L27 | AF2 | KRAS G12C |
| L27 | AF3 | KRAS G12R |
| L27 | AF4 | KRAS G12S |
| L28 | AF | EGFR E709G |
| L28 | AF2 | EGFR E709V |
| L28 | AF3 | EGFR E709A |
| L28 | AF4 | WT |
| L29 | AF | WT |
| L29 | AF2 | NRAS Q61K |
| L29 | AF3 | NRAS Q61E |
| L29 | AF4 | NRAS Q61L |
| L30 | AF | PIK3CA M1043I |
| L30 | AF2 | PIK3CA M1043I |
| L30 | AF3 | WT |
| L30 | AF4 | PIK3CA M1043I |
| L31 | AF | ALK L1196V |
| L31 | AF2 | ALK L1196L |
| L31 | AF3 | WT |
| L31 | AF4 | ALK L1196M |
| L32 | AF | EGFR H835P |
| L32 | AF2 | WT |
| L32 | AF3 | EGFR H835R |
| L32 | AF4 | EGFR H835L |
| L33 | AF | WT |
| L33 | AF2 | EGFR del |
| L33 | AF3 | EGFR I744M |
| L33 | AF4 | EGFR ins |
| L34 | AF | EGFR del |
| L34 | AF2 | EGFR E746_T751>S |
| L34 | AF3 | EGFR del |
| L34 | AF4 | WT |
| L35 | AF | MAP2K1 D67H |
| L35 | AF2 | MAP2K1 D67N |
| L35 | AF3 | WT |
| L35 | AF4 | MAP2K1 D67Y |
| L36 | AF | WT |
| L36 | AF2 | \ |
| L36 | AF3 | \ |
| L36 | AF4 | EGFR ins |
| L37 | AF | NRAS Q61R |
| L37 | AF2 | NRAS Q61L |
| L37 | AF3 | NRAS Q61P |
| L37 | AF4 | WT |
| L38 | AF | EGFR G719R |
| L38 | AF2 | EGFR G719S |
| L38 | AF3 | WT |
| L38 | AF4 | EGFR G719C |
| L39 | AF | BRAF D594G |
| L39 | AF2 | BRAF D594V |
| L39 | AF3 | BRAF D594A |
| L39 | AF4 | WT |
| L40 | AF | WT |
| L40 | AF2 | BRAF G466V |
| L40 | AF3 | BRAF G466A |
| L40 | AF4 | BRAF G466E |
| L41 | AF | MAP2K1 Q56R |
| L41 | AF2 | MAP2K1 Q56L |
| L41 | AF3 | MAP2K1 Q56P |
| L41 | AF4 | WT |
| L42 | AF | \ |
| L42 | AF2 | EGFR del |
| L42 | AF3 | WT |
| L42 | AF4 | EGFR del |
| L43 | AF | EGFR G719A |
| L43 | AF2 | EGFR G719D |
| L43 | AF3 | WT |
| L43 | AF4 | EGFR G719A |
| L44 | AF | EGFR ins |
| L44 | AF2 | EGFR ins |
| L44 | AF3 | WT |
| L44 | AF4 | EGFR V774L |
| L45 | AF | EGFR L858P |
| L45 | AF2 | EGFR L858Q |
| L45 | AF3 | EGFR L858R |
| L45 | AF4 | WT |
| L46 | AF | KRAS Q61E |
| L46 | AF2 | KRAS Q61* |
| L46 | AF3 | WT |
| L46 | AF4 | KRAS Q61K |
| L47 | AF | EGFR S752_I759delSPKANKEI |
| L47 | AF2 | WT |
| L47 | AF3 | \ |
| L47 | AF4 | \ |
| L48 | AF | NRAS Q61Q |
| L48 | AF2 | NRAS Q61H |
| L48 | AF3 | NRAS Q61H |
| L48 | AF4 | WT |
| L49 | AF | BRAF V600A |
| L49 | AF2 | BRAF V600E |
| L49 | AF3 | BRAF V600G |
| L49 | AF4 | WT |
| L50 | AF | DDR2 L239P |
| L50 | AF2 | DDR2 L239Q |
| L50 | AF3 | DDR2 L239R |
| L50 | AF4 | WT |
| L51 | AF | PIK3CA E542Q |
| L51 | AF2 | PIK3CA E542K |
| L51 | AF3 | WT |
| L51 | AF4 | PIK3CA E542* |
| L52 | AF | EGFR N771>TH |
| L52 | AF2 | WT |
| L52 | AF3 | EGFR ins |
| L52 | AF4 | EGFR D770_N771insMATP |
| L53 | AF | EGFR T854A |
| L53 | AF2 | EGFR T854S |
| L53 | AF3 | EGFR T854P |
| L53 | AF4 | WT |
| L54 | AF | NRAS G12R |
| L54 | AF2 | NRAS G12S |
| L54 | AF3 | WT |
| L54 | AF4 | NRAS G12C |
| L55 | AF | EGFR L747_P753>Q |
| L55 | AF2 | \ |
| L55 | AF3 | EGFR L747_E749delLRE |
| L55 | AF4 | WT |
| L56 | AF | KRAS Q61R |
| L56 | AF2 | KRAS Q61L |
| L56 | AF3 | KRAS Q61P |
| L56 | AF4 | WT |
| L57 | AF | BRAF G469A |
| L57 | AF2 | BRAF G469E |
| L57 | AF3 | WT |
| L57 | AF4 | BRAF G469V |
| L58 | AF | KRAS G13A |
| L58 | AF2 | KRAS G13D |
| L58 | AF3 | WT |
| L58 | AF4 | KRAS G13V |
| L59 | AF | DDR2 S768S |
| L59 | AF2 | DDR2 S768R |
| L59 | AF3 | DDR2 S768R |
| L59 | AF4 | WT |
| L60 | AF | RET M918T |
| L60 | AF2 | \ |
| L60 | AF3 | \ |
| L60 | AF4 | WT |
| L61 | AF | MAP2K1 K57N |
| L61 | AF2 | MAP2K1 K57K |
| L61 | AF3 | WT |
| L61 | AF4 | MAP2K1 K57N |
| L62 | AF | EGFR V769_D770insASV |
| L62 | AF2 | WT |
| L62 | AF3 | EGFR ins |
| L62 | AF4 | \ |
| L63 | AF | EGFR E746_A750delELREA (2236_2250del) |
| L63 | AF2 | E746_A750>VP |
| L63 | AF3 | \ |
| L63 | AF4 | WT |
| L64 | AF | WT |
| L64 | AF2 | ALK C1156F |
| L64 | AF3 | ALK C1156S |
| L64 | AF4 | ALK C1156Y |
| L65 | AF | EGFR V774_C775insHV |
| L65 | AF2 | EGFR ins |
| L65 | AF3 | WT |
| L65 | AF4 | \ |
| L66 | AF | ERBB2 A775A |
| L66 | AF2 | ERBB2 ins |
| L66 | AF3 | ERBB2 A775A |
| L66 | AF4 | WT |
| L67 | AF | \ |
| L67 | AF2 | EGFR E746_A750delELREA (2235_2249del) |
| L67 | AF3 | WT |
| L67 | AF4 | \ |
| L68 | AF | EGFR T790R |
| L68 | AF2 | EGFR T790M |
| L68 | AF3 | WT |
| L68 | AF4 | EGFR T790K |
| L69 | AF | WT |
| L69 | AF2 | KRAS G13C |
| L69 | AF3 | KRAS G13R |
| L69 | AF4 | KRAS G13S |
| L70 | AF | EGFR ins |
| L70 | AF2 | EGFR S768N |
| L70 | AF3 | WT |
| L70 | AF4 | EGFR S768I |
| L71 | AF | ERBB2 ins |
| L71 | AF2 | ERBB2 G776S |
| L71 | AF3 | WT |
| L71 | AF4 | ERBB2 ins |

## 3.5 Ion Torrent™ Genexus™ Integrated Sequencer System

To perform retrospective re-evaluation of cases tested negative with Sequenom MassARRAY® and enrolling new cases of ADKs, an Ion Torrent™ Genexus™ Integrated Sequencer System, using Oncomine™ Precision Assay GX (DNA panel), associated with Ion Torrent GX5 Chip (ThermoFisher Scientific, Milan, Italy) has been used. Genexus is a fully automated system: it is possible to test mutations (45 hot-spots) and copy number variations (14 CNV genes) across 50 genes (listed in Table 2), starting form 10ng/µl of DNA input (Ion Torrent™ AmpliSeq™ HD Technology), allowing to test 32 sample each run. This system is ideal for fast genomic profiling in clinical cancer.

*Table 2: Oncomine Precision Assay (OPA) included genes.*

| |
|---|
| DNA hot-spots: |
| *AKT1, AKT2, AKT3, ALK, AR, ARAF, BRAF, CDK4, CDKN2A, CHEK2, CTNNB1, EGFR, ERBB2, ERBB3, ERBB4, ESR1, FGFR1, FGFR2, FGFR3, FGFR4, FLT3, GNA11, GNAQ, GNAS, HRAS, IDH1, IDH2, KIT, KRAS, MAP2K1, MAP2K2, MET, MTOR, NRAS, NTRK1, NTRK2, NTRK3, PDGFRα, PIK3CA, PTEN, RAF1, RET, ROS1, SMO, TP53.* |
| Copy Number Variation: |
| *ALK, AR, CD274, CDKN2A, EGFR, ERBB2, ERBB3, FGFR1, FGFR2, FGFR3, KRAS, MET, PIK3CA, PTEN.* |

Results were analyzed with Ion Torrent Genexus™ Software 6.6. Through this software in Cloud is possible to program the run, form a minimum of 8 sample to a maximum of 32.

## 3.6 Real-Time PCR

### *3.6.1 EasyPGX®ALK\ROS\MET\RET*

RNAs were analyzed by means of CE-IVD EasyPGX®ALK\ROS\MET\RET (Diatechpharmacogenetics, Jesi, Italy) to detect the main chromosomal translocations involving *ALK, ROS1, RET* genes and the *MET* exon 14 skipping mutation in One Step Real-Time PCR. Each mix allows the co-amplification of one or more mutated alleles plus an endogenous control gene (limit of detection is 0.5%). This test was performed on all FFPE biopsies and tissue samples that we were able to collect and obtained RNA in acceptable quality and quantity. Samples have been

amplified by means of Agilent EasyPGX® and results have been analyzed with both Agilent AriaDX 4.1 and EasyPGX Analysis 4.0.9 softwares. (Figure 2)

*Figure 2: Gene fusions and skipping mutations list detectable by the RT-PCR*

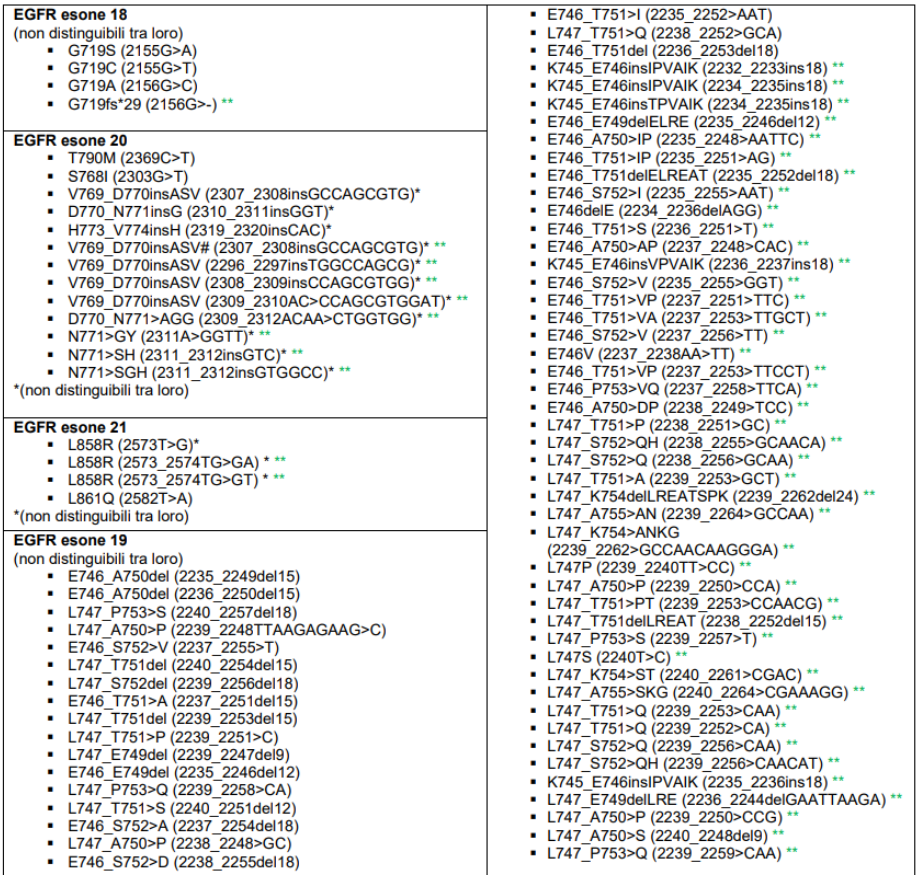
| |
|---|
| **ALK**<br>▪ Valutazione dello sbilanciamento delle porzioni 5P e 3P dell'mRNA *wild-type* di *ALK* generato da eventi di fusioni geniche. |
| **ROS1 exon 32** (non distinguibili tra loro)<br>▪ SLC34A2-ROS1 S4:R32 (RefSeq - EU236946)<br>▪ SLC34A2-ROS1 S13del2046:R32 (RefSeq - AB795243)<br>▪ CD74-ROS1 C6:R32 (RefSeq - AB795244)<br>▪ SDC4-ROS1 S2:R32 (RefSeq - AB795240)<br>▪ SDC4-ROS1 S4:R32 (COSMIC v76 - COSF1279) |
| **ROS1 exon 34** (non distinguibili tra loro)<br>▪ SLC34A2-ROS1 S4:R34 (RefSeq - EU236947)<br>▪ SLC34A2-ROS1 S13del2046:R34 (PMID - 22327623)<br>▪ CD74-ROS1 C6:R34 (PMID - 18083107)<br>▪ SDC4-ROS1 S4:R34 (RefSeq - AB795242)<br>▪ EZR-ROS1 E10:R34 (RefSeq - AB795246) |
| **ROS1 exon 35-36** (non distinguibili tra loro)<br>▪ LRIG3-ROS1 L16:R35 (RefSeq - AB795248)<br>▪ TPM3-ROS1 T8:R35 (RefSeq - AB795239)<br>▪ (FIG)GOPC-ROS1 G8:R35 (PMID - 22661537)<br>▪ (FIG)GOPC-ROS1 G4:R36 (PMID - 22661537) |
| **RET exon 12** (non distinguibili tra loro)<br>▪ CCDC6-RET C1:R12 (RefSeq - AB698668)<br>▪ KIF5B-RET K15:R12 (RefSeq - AB795250)<br>▪ KIF5B-RET K16:R12 (RefSeq - AB795252)<br>▪ KIF5B-RET K22:R12 (RefSeq - AB795254)<br>▪ KIF5B-RET K23:R12 (RefSeq - AB795256)<br>▪ NCOA4-RET N7:R12 (PMID - 23150706) |
| **RET exon 8-11** (non distinguibili tra loro)<br>▪ KIF5B-RET K15:R11 (PMID - 22327622)<br>▪ KIF5B-RET K24:R11 (RefSeq - AB795257)<br>▪ KIF5B-RET K24:R8 (PMID - 22327624) |
| **MET exon 14**<br>▪ MET Exon 14 skipping |

### *3.6.2 Easy®EGFR*

ctDNAs extracted from plasma have been analyzed using a CE-IVD commercial kit Easy®EGFR (Diatechpharmacogenetics, Jesi, Italy). The kit contains 85 main mutations in *EGFR* exons 18, 19, 20 and 21 by means of 8 master mix of oligonucleotides. Each oligonucleotide mixture allows the co-amplification of one or more mutated alleles plus an endogenous control gene. A specific mixture of control oligonucleotides allows the evaluation of the quality and quantity of the DNA present in the samples. A control standard positive DNA for all mixes is also tested in parallel. It allows to detect low percentages of mutated allele in the presence of high quantities of wild type genomic DNA by real time amplification with sequence specific probes marked with fluorophores (limit of detection is 0.5%). Results have been amplified by means of Rotor-Gene™ 600 (Qiagen, Hilden, Germany) and analyzed with Rotor-Gene 6000 Series Software. This test was performed on

all the liquid biopsies that we were able to collect in pre-established timings also based on the needs and the state of health of patients; whether we had tissue material available, so as not to have to re-biopsy the patient, or in case of lack of tissue material.(Figure 3)

*Figure 3: EGFR detection probes*



**EGFR esone 18**
(non distinguibili tra loro)
- G719S (2155G>A)
- G719C (2155G>T)
- G719A (2156G>C)
- G719fs*29 (2156G>-) **

**EGFR esone 20**
- T790M (2369C>T)
- S768I (2303G>T)
- V769_D770insASV (2307_2308insGCCAGCGTG)*
- D770_N771insG (2310_2311insGGT)*
- H773_V774insH (2319_2320insCAC)*
- V769_D770insASV# (2307_2308insGCCAGCGTG)* **
- V769_D770insASV (2296_2297insTGGCCAGCG)* **
- V769_D770insASV (2308_2309insCCAGCGTGG)* **
- V769_D770insASV (2309_2310AC>CCAGCGTGGAT)* **
- D770_N771>AGG (2309_2312ACAA>CTGGTGG)* **
- N771>GY (2311A>GGTT)* **
- N771>SH (2311_2312insGTC)* **
- N771>SGH (2311_2312insGTGGCC)* **

*(non distinguibili tra loro)

**EGFR esone 21**
- L858R (2573T>G)*
- L858R (2573_2574TG>GA) * **
- L858R (2573_2574TG>GT) * **
- L861Q (2582T>A)

*(non distinguibili tra loro)

**EGFR esone 19**
(non distinguibili tra loro)
- E746_A750del (2235_2249del15)
- E746_A750del (2236_2250del15)
- L747_P753>S (2240_2257del18)
- L747_A750>P (2239_2248TTAAGAGAAG>C)
- E746_S752>V (2237_2255>T)
- L747_T751del (2240_2254del15)
- L747_S752del (2239_2256del18)
- E746_T751>A (2237_2251del15)
- L747_T751del (2239_2253del15)
- L747_T751>P (2239_2251>C)
- L747_E749del (2239_2247del9)
- E746_E749del (2235_2246del12)
- L747_P753>Q (2239_2258>CA)
- L747_T751>S (2240_2251del12)
- E746_S752>A (2237_2254del18)
- L747_A750>P (2238_2248>GC)
- E746_S752>D (2238_2255del18)
- E746_T751>I (2235_2252>AAT)
- L747_T751>Q (2238_2252>GCA)
- E746_T751del (2236_2253del18)
- K745_E746insIPVAIK (2232_2233ins18) **
- K745_E746insIPVAIK (2234_2235ins18) **
- K745_E746insTPVAIK (2234_2235ins18) **
- E746_E749delELRE (2235_2246del12) **
- E746_A750>IP (2235_2248>AATTC) **
- E746_T751>IP (2235_2251>AG) **
- E746_T751delELREAT (2235_2252del18) **
- E746_S752>I (2235_2255>AAT) **
- E746delE (2234_2236delAGG) **
- E746_T751>S (2236_2251>T) **
- E746_A750>AP (2237_2248>CAC) **
- K745_E746insVPVAIK (2236_2237ins18) **
- E746_S752>V (2235_2255>GGT) **
- E746_T751>VP (2237_2251>TTC) **
- E746_T751>VA (2237_2253>TTGCT) **
- E746_S752>V (2237_2256>TT) **
- E746V (2237_2238AA>TT) **
- E746_T751>VP (2237_2253>TTCCT) **
- E746_P753>VQ (2237_2258>TTCA) **
- E746_A750>DP (2238_2249>TCC) **
- L747_T751>P (2238_2251>GC) **
- L747_S752>QH (2238_2255>GCAACA) **
- L747_S752>Q (2238_2256>GCAA) **
- L747_T751>A (2239_2253>GCT) **
- L747_K754delLREATSPK (2239_2262del24) **
- L747_A755>AN (2239_2264>GCCAA) **
- L747_K754>ANKG (2239_2262>GCCAACAAGGGA) **
- L747P (2239_2240TT>CC) **
- L747_A750>P (2239_2250>CCA) **
- L747_T751>PT (2239_2253>CCAACG) **
- L747_T751delLREAT (2238_2252del15) **
- L747_P753>S (2239_2257>T) **
- L747S (2240T>C) **
- L747_K754>ST (2240_2261>CGAC) **
- L747_A755>SKG (2240_2264>CGAAAGG) **
- L747_T751>Q (2239_2253>CAA) **
- L747_T751>Q (2239_2252>CA) **
- L747_S752>Q (2239_2256>CAA) **
- L747_S752>QH (2239_2256>CAACAT) **
- K745_E746insIPVAIK (2235_2236ins18) **
- L747_E749delLRE (2236_2244delGAATTAAGA) **
- L747_A750>P (2239_2250>CCG) **
- L747_A750>S (2240_2248del9) **
- L747_P753>Q (2239_2259>CAA) **

** *mutations must be analyzed by in silico PCR and sequenced*

## 3.7 Immunohistochemistry and Fluorescence in situ hybridization

These traditional methods were used exclusively for all those patients from whom it was not possible to extract RNA due to the scarcity of tissue material available.

### *3.7.1 Immunohistochemistry*

We used the rabbit monoclonal Antibody (mAb) anti-ALK D5F3 clone to evaluate the possible presence of ALK expression using the Ventana immuno-stainer instruments (Roche, Basel, Switzerland) as manufacturer's instructions starting from 2µm FFPE tissue sections.

ALK immunostainig have been evaluated with the following score:[132]

- 3+ Strong cytoplasmatic granular staining
- 2+ Moderate cytoplasmatic staining – borderline
- 1+ Weak cytoplasmatic staining in at least 10% of tumour cells.
- 0 Negative

### *3.7.2 Fluorescence in situ hybridization*

A completely manual FISH protocol for the evaluation of rearrangements of the *ALK*, *ROS1* and *RET* genes has been used. Probes mixed used are Zytolight® SPEC ALK (*locus* 2p23), Zytolight® SPEC ROS1 (*locus* 6q22) and Zytolight® SPEC RET (*locus* 10q11.21) Dual Color Break Apart Probe (ZytoVision GmbH – Bremerhaven – Germany).

Break-Apart probes are designed to detect translocation involving the chromosomal region of interest that harbors a specific gene. *ALK*, *ROS1* and *RET* probe mix are mapped below.(Figure 4).

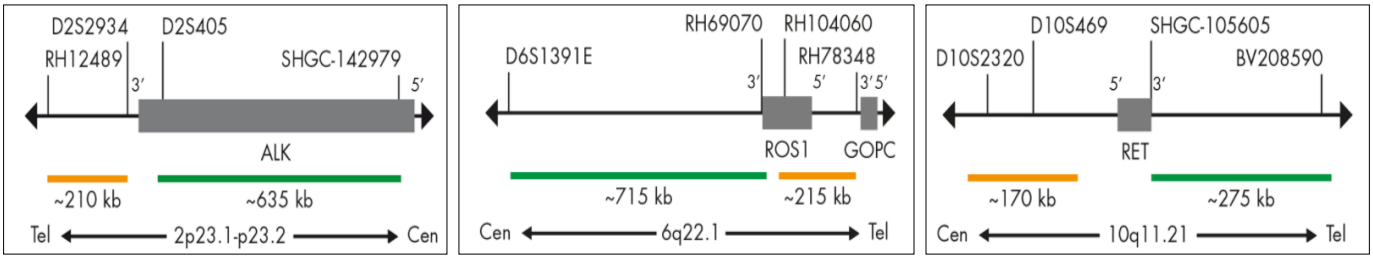



*Figure 4: SPEC ALK, ROS1 and RET probes*

Using a Leica DM 5500 B Fluorescence microscope (Leica Microsystem SRL, Milan, Italy), histological samples for each single case were evaluated and interpreted as follow:

- normal interphase cells are indicated by two orange/green fusion signals per nucleus; tumor cells with translocations affecting these loci as indicated with ore orange/green fusion signal (non-rearranged) one orange and one separated green signal as a standard translocation pattern.[133]
- *ROS1* amplification: FISH is considered positive with ROS1 amplification: a) >40% of tumour cells with a mean of ≥4 *ROS1* signal copies per nucleus; b) *ROS1*/CEP6 ratio ≥2; c) 10% of tumour cells with a mean of ≥15 *ROS1* signals copies per nucleus. *ROS1* amplification is associated at poor prognosis; there are no evidences of targatale drug benefit.
- *RET* amplification: FISH is considered positive with the presence of a mean of ≥5 RET signals copies in tumour cells.[134]

Also the presence of an extra signal at the 3' of each genes studied, corresponds to the activation of the tyrosine kinase domain, conferring an imbalanced translocation.

FISH protocol reported below starting from 4µm FFPE tissue sections.

**Dewax:**

- Xilolo 2 x 10' RT
- EtOH 100% 2 x 5' RT
- Led slides dry

**Pre-treatment:**

- Pretreatment Solution (PS) 30' , 80°C
- $H_2O$ 1' RT

**Protease Treatment:**

- Protease Buffer (PB)* + Protease (PBP)* 5' -15', 37°C
- Washing Buffer I pH 7-7,5 5' RT
- Washing Buffer II pH 7-7,5 5' RT
- Check tissue digestion at microscope

**Hybridation:**

- EtOH 70-85-100% 2' RT in each one
- Add 10µl probe and seal off with a 22x22 mm coverslide and rubbercement.
- Incubation overnight in Hybrite 5' , 85°C (co-debaturation time)
  O/N , 37°C (hybridation time)

**Post-Hybridation Washing:**

- Remove rubbercement and cover slide gently
- 0,4 x SSC/ 0,3% NP40 3' RT
- 0,4 x SSC/ 0,3% NP40 2' , 72°C
- 0,4 x SSC/ 0,3% NP40 5''-1' RT
- Mount slides with DAPI II counter dye with antifade.
- Keep slides at -20°C.

Fluorescence microscope is linked with a PC and interfaced with CytoVision software (supplied by Leica Microsystem SRL, Milan, Italy), which allows the acquisition of images through an RGB camera.

## 3.8 Sanger Sequencing

To study *MET* gene exon 14 skipping mutation *status*, different PCRs have been performed. For each case, three pairs of primers were constructed covering intron region 13-14 and the entire exon 14, using *PRIMER3* and *USCS Genome Browser Bioinformatic*, and verified products with *in silico* PCR.[135] (Table 3)

Standardized PCR conditions used were the follow: 10XB $[1X]_f$, $MgCl_2$ $[1,5mM]_f$ e Ampli-Taq Gold $[1\ U/campione]_f$ (ThermoFisher Scientific, Milan, Italy) and dNTPs $[200\mu M]_f$ (Roche, Milan, Italy), Home made primers $[10pmoli/\mu l]_f$.

Thermal profile has been optimized using VerityPro Thermal Cycler (ThermoFisher Scientific, Milan, Italy).

*Table 3: cMET gene oligonucleotides sequence*

| Gene | | Primer Forward (5'-3') | Primer Reverse (3'-5') | PCR Product |
|---|---|---|---|---|
| ***MET*** | **1** | CATGAGTTCTGGGCACTGGG | TAGTTGGGCTTACACTTCGGG | 182pb |
| | **2** | AGGCTTGTAAGTGCCCGAAG | CAATGTCACAACCCACTGAGG | 210pb |
| | **3** | TGCTGGTGTTGTCTCAATATCA | AGACTTTGACCCAGTGCCC | 250pb |

PCR products were verified with 2% agarose gel (Gellyphore - CELBIO)(BIORAD Subcell GT Laboratory Supplies) in TAE 1X buffer (2M Tris-Acetate, 0.05M EDTA Eppendorf).

DNA was stained with ethidium bromide, observed under UV rays and finally photographed. A molecular weight marker has been used every electrophoretic run to evaluate PCR fragments size (Marker V, Roche).

PCR products were purified with Illustra™ Exostar™ 1Step (GE HealthCare LifeScience - Amersham Place Little Chalfont, Buckinghamshire – UK).

- 2µl Illustra™ Exostar™ + 5µl PCR products
- Incubation: 37°C, 15’ and 80°C, 15° on VerityPro Thermal Cycler (ThermoFisher Scientific, Milan, Italy)
- Keep purified PCR product -20°C.

Sequencing PCR: amplified fragments were labeled with a dNTPs and ddNTPs fluorescinated pool, using BigDye Terminator v1.1 Ready Reaction Cycle Sequencing kit (ThermoFisher Scientific, Milan, Italy) following manufacturer’s instructions:

Master Mix:

- ddNTPs + dNTPs pool with
- AmpliTaq DNA polymerase

} BigDye Terminator v 1.1

- $MgCl_2$ 2mM
- Tris-HCl 80mM, pH 9.0

} 5X Sequencing Buffer

- Add 1µl of purified product for Sequencing PCR
- $H_2O$ DEPC

Sequencing PCRs were performed with VerityPro Thermal Cycler (ThermoFisher Scientific, Milan, Italy) using an optimized thermal profile. Labeled fragments were than purified by means of X-Terminator Beads (ThermoFisher Scientific, Milan, Italy) and analyzed with SeqStudio™ Genetic Analyzer (4-capillary) (ThermoFisher Scientific, Milan, Italy).

DNA sequencies obtained were studied with *Chromas* v.*1.45* software and aligned in FASTA format with wild-type sequence in *BLAST* (Basic Local Alignment Search Tool) database.

# 4. RESULTS

## 4.1 Patients Clinical Characteristics

During the pandemic, a far greater number of imaging tests, especially CT-scans, were performed than in normal conditions. This also made it possible to identify many early-stage tumors, as well as perform an initial diagnosis of the presence/absence of a viral COVID-19 infection in the thoracic area. Specifically, our multidisciplinary group has set itself the goal of treating/monitoring, where possible, all ADKs, especially for those patients also affected by COVID-19.

Between March 2020 and February 2021, 1447 cases of ADKs were isolated and analyzed by means of histological anatomic pathology procedures and classified in different cancer subtypes and stages: 23.3% presented an early cancer stage, while the remaining part presented stage II or III-IV; a part of them have come to the attention of our center not because they were affected by COVID-19, but because they are included in standard monitoring and diagnosing cancer processes. All the other patients were affected by COVID-19, if virological clinical situation allowed, were also monitored for lung tumor. (Table 4)

Moreover new 570 patients (143 stage I, 98 stage II, 329 stages III and IV), enrolled in the study have been evaluated from a molecular point of view with this method. Thus bringing the total population of the study cohort to 2017 patients. (Table 5)

At the end of 2021, patients were 1121 male and 896 female, from age 33 years to age 96 years (mean 64.4 years, median 64.5 years). The 72% was affected by COVID-19. Often, the situation relating to viral infection prevented patients from continuing with anti-cancer treatments, once the therapeutic profile was outlined. Among the studied patients, COVID-19 infection was fatal mainly in advanced ADKs, where the organism was obviously more debilitated, with a mortality rate of 39.5%. In other ADKs, COVID-19 mortality rate was 28.3%.

*Table 4: Patient classification at "Time 0" in 2020*

| Patients number | Cancer Stage | COVID-19 + ADK | ADK only |
|---|---|---|---|
| **338** | I | 310 | 28 |
| **285** | II | 199 | 86 |
| **535** | III | 310 | 225 |
| **327** | IV | 142 | 185 |
| **Total 1447** | | **961** | **524** |

*Table 5: New patient classification in 2021*

| Patients number | Cancer Stage | COVID-19 + ADK | ADK only |
|---|---|---|---|
| **338 + 143** | I | 310 + 134 | 28 + 9 |
| **285 + 98** | II | 199 + 76 | 86 + 22 |
| **535 + 212** | III | 310 + 184 | 225 + 28 |
| **327 + 117** | IV | 142 + 93 | 185 + 24 |
| **Total 1447 + 570** | | **961 + 418** | **524 + 83** |

In 18 cases it was not possible perform tissue biopsy for the anatomic position of tumor and/or patient general conditions, so it has been decided to monitor them only by means of liquid biopsy.
Liquid biopsy is also used to follow-up more cases as possible (with patients compliance and good health conditions), in different and pre-defined timings. 1516 liquid biopsies have been collected and analyzed from March 2020 to December 2021.

## 4.2 Histology Classification

All cases were studied and classified from clinical, immunophenotype and molecular evidences, based on WHO Classification of tumors of the lung, pleura thymus and heart 2015.[108]
At the beginning of the year 2022 the draft of the WHO 2021 was made, in which, it would seem, the histological and immunophenotypic criteria have not been changed compared to the previous classification. However, the novelties of the new WHO are mostly of a molecular-genetic field, which describes the new methods that could be adopted by molecular pathology laboratories as the gold standard for diagnostics. From morphological analysis of the histological tissue samples, a fairly heterogeneous picture emerged as regards the composition of ADKs cases observed. In particular the cohort presented mucinous and non-mucinous type tumours, with single and/or mixed type (28% of cases).
The acinar pattern was prevalent in mixed cases (44.4%) and in pure cases (13.6%). The non-mucinous lepidic pattern was present in 10% of cases of the mixed type, the micro-papillary in 2% of patients, of the mixed type and in 1% in pure form. Of the mucinous cases, a few cases presented diffuse type (3%), with a lepidic component, and others polyadenoid-cribriform, with a limited component of signet ring cells.(Table 6)

*Table 6: Enrolled patients clinical and histological characteristics in 2020*

| Patients ADK only | COVID-19 (affected patients) | Diagnosis (WHO 2015) | Grade | Stage |
|---|---|---|---|---|
| 5 | 21 | Misto: acinar (70%) and lepidic (30%) | 2 | IA |
| 4 | 39 | Mixed: papillary (50%), lepidic (30%) and acinar (20%) | 2 | IA |
| 5 | 19 | Acinar | 2 | IA |
| 4 | 50 | Mixed: acinar (80%) and micropapillary (20%) | 2 | IA |
| 6 | 25 | Acinar | 1 | IA |
| 4 | 38 | Mixed: acinar (75%) and lepidic (25%) | 1 | IA |
| 19 | 32 | Mixed: papillary (70%) and lepidic (30%) | 1 | IA |
| 16 | 23 | Micropapillary | 2 | IA |
| 8 | 15 | Mixed: acinar (90%) and micropapillary (10%) | 2 | IB |
| 9 | 18 | Mixed: acinar (50%) and micropapillary (50%) | 2 | IIA |
| 23 | 35 | Misto: acinar (70%) and lepidic (30%) | 2 | IIA |
| 26 | 42 | Mixed: lepidic (80%) and micropapillary (20%) | 2 | IIA |
| 26 | 18 | Mixed: acinar (80%) and solid (20%) | 2 | IIA |
| 11 | 11 | Solid with mucus | 3 | IIA |
| 15 | 29 | Acinar | 2 | IIA |
| 10 | 43 | Solid with mucus | 3 | IIIA |
| 24 | 47 | Acinar | 3 | IIIA |
| 16 | 33 | Mucinous with lepidic mucinous fraction component | 2 | IIIA |
| 22 | 42 | Mixed: micropapillary (70%) and acinar (30%) | 2 | IIIA |
| 24 | 42 | Mixed: acinar (90%) and micropapillary (10%) | 3 | IIIA |
| 24 | 31 | Mixed: acinar (50%) and micropapillary (50%) with mucus and signet ring cells | 2 | IIIB |
| 19 | 34 | Mixed: acinar (50%) and micropapillary (50%) | 3 | IIIB |
| 22 | 19 | Micropapillary | 3 | IIIB |
| 29 | 54 | Mucinous with lepidic mucinous fraction component | 3 | IIIB |
| 11 | 12 | Solid with mucus | 3 | IVA |
| 9 | 10 | Acinar | 3 | IVA |
| 13 | 27 | Mucinous with lepidic mucinous fraction component | 3 | IVA |
| 5 | 19 | Mixed: micropapillary (75%) and acinar (30%) | 3 | IVA |
| 20 | 48 | Mixed: acinar (80%) and micropapillary (25%) | 3 | IVB |
| 19 | 30 | Mixed: acinar (65%) and micropapillary (45%) with mucus and signet ring cells | 3 | IVB |
| 6 | 9 | Mixed: acinar (50%) and micropapillary (50%) | 3 | IVB |
| 12 | 22 | Micropapillary | 3 | IVB |
| 31 | 24 | Mucinous with lepidic mucinous fraction component | 3 | IVB |

## 4.3 Mutational Analyses

DNA and RNA were isolated as previously described, using specific protocols. Nucleic acids were extracted *ad hoc* to be used for subsequent molecular analyses. Each procedure in fact provides for the quantity and quality of the sample to obtain a result with clinical-diagnostic value.

In particular, the extraction and purification procedures of nucleic acids have been optimized to be used with a transversal manner on laboratory several methods. From a technical point of view, there were no problems in the isolation and treatment of the biological material; DNA and RNA were of good quality, even in COVID-19 patients where the starting material had much more presence of fibrotic or necrotic tissue compared with a neoplastic lung tissue. Compared to a patient affected only by neoplasia, for the same material, the yields of nucleic acids were slightly lower, but not in quality.

### *4.3.1 SEQUENOM® MassARRAY®*

From March 2020 to February-March 2021, 1447 patients affected of lung ADKs have been analyzed by means of MS. Results obtained are in line with those described in main articles published in literature.[136] Even in patients with stage I and II cancer (585 patients), it was possible to find the presence of driver mutations in 11.7% of cases (69/585 patients), as described in the Table 7 below. In Table 8 are reported results for patients stage III and IV were positive for driver and targetable mutations, at the percentage of 23.6%;

*Table 7: Mutated patients stage I and II by means of MS*

| Patients ADK only | COVID-19 Affected Patients | Diagnosis (WHO 2015) | Grade | Stage | Mutated patients | Mutated patients (COVID-19) |
|---|---|---|---|---|---|---|
| 5 | 21 | Misto: acinar (70%) and lepidic (30%) | 2 | IA | 3 | 1 |
| 4 | 39 | Mixed: papillary (50%), lepidic (30%) and acinar (20%) | 2 | IA | 0 | 7 |
| 5 | 19 | Acinar | 2 | IA | 1 | 4 |
| 4 | 50 | Mixed: acinar (80%) and micropapillary (20%) | 2 | IA | 1 | 7 |
| 6 | 25 | Acinar | 1 | IA | 0 | 2 |
| 4 | 38 | Mixed: acinar (75%) and lepidic (25%) | 1 | IA | 0 | 7 |
| 19 | 32 | Mixed: papillary (70%) and lepidic (30%) | 1 | IA | 2 | 3 |
| 16 | 23 | Micropapillary | 2 | IA | 1 | 3 |
| 8 | 15 | Mixed: acinar (90%) and micropapillary (10%) | 2 | IB | 0 | 1 |
| 9 | 18 | Mixed: acinar (50%) and micropapillary (50%) | 2 | IIA | 1 | 1 |
| 23 | 35 | Misto: acinar (70%) and lepidic (30%) | 2 | IIA | 1 | 5 |
| 26 | 42 | Mixed: lepidic (80%) and micropapillary (20%) | 2 | IIA | 3 | 3 |
| 26 | 18 | Mixed: acinar (80%) and solid (20%) | 2 | IIA | 1 | 3 |
| 11 | 11 | Solid with mucus | 3 | IIA | 2 | 0 |
| 15 | 29 | Acinar | 2 | IIA | 2 | 4 |

*Table 8: Mutated patients stage III and IV by means of MS*

| Patients ADK only | COVID-19 Affected Patients | Diagnosis (WHO 2015) | Grade | Stage | Mutated patients | Mutated patients (COVID-19) |
|---|---|---|---|---|---|---|
| 10 | 43 | Solid with mucus | 3 | IIIA | 2 | 8 |
| 24 | 47 | Acinar | 3 | IIIA | 4 | 19 |
| 16 | 33 | Mucinous with lepidic mucinous fraction component | 2 | IIIA | 5 | 3 |
| 22 | 42 | Mixed: micropapillary (70%) and acinar (30%) | 2 | IIIA | 2 | 7 |
| 24 | 42 | Mixed: acinar (90%) and micropapillary (10%) | 3 | IIIA | 4 | 14 |
| 24 | 31 | Mixed: acinar (50%) and micropapillary (50%) with mucus and bezel ring cells | 2 | IIIB | 2 | 9 |
| 19 | 34 | Mixed: acinar (50%) and micropapillary (50%) | 3 | IIIB | 3 | 10 |
| 22 | 19 | Micropapillary | 3 | IIIB | 2 | 4 |
| 29 | 54 | Mucinous with lepidic mucinous fraction component | 3 | IIIB | 9 | 23 |
| 11 | 12 | Solid with mucus | 3 | IVA | 2 | 4 |
| 9 | 10 | Acinar | 3 | IVA | 1 | 3 |
| 13 | 27 | Mucinous with lepidic mucinous fraction component | 3 | IVA | 4 | 10 |
| 5 | 19 | Mixed: micropapillary (75%) and acinar (30%) | 3 | IVA | 0 | 2 |
| 20 | 48 | Mixed: acinar (80%) and micropapillary (25%) | 3 | IVB | 3 | 14 |
| 19 | 30 | Mixed: acinar (65%) and micropapillary (45%) with mucus and bezel ring cells | 3 | IVB | 4 | 8 |

### *4.3.2 Ion Torrent™ Genexus™ Integrated Sequencer System*

The introduction of NGS instrument gave the possiblity to review 1174 cases that resulted negative for MS.

In particular, the presence of driver mutations with an allelic frequency <5% were found in 20% of early stage patients who tested negative for MS. (Table 9)

Also cases stage III-IV found negative were re-evaluated in NGS: we found 109/658, 16.5% more patients positive for driving mutation with <5% of allelic frequency. (Table 10)

*Table 9: Re-Tested wild-type patients stage I and II: new mutation found with <5% allelic frequency by means of NGS*

| Patients ADK only | COVID-19 Affected Patients | Diagnosis (WHO 2015) | Grade | Stage | Mutated patients | Mutated patients COVID-19 |
|---|---|---|---|---|---|---|
| 2 | 20 | Misto: acinar (70%) and lepidic (30%) | 2 | IA | 1 | 9 |
| 4 | 32 | Mixed: papillary (50%), lepidic (30%) and acinar (20%) | 2 | IA | 2 | 16 |
| 4 | 15 | Acinar | 2 | IA | 1 | 6 |
| 3 | 43 | Mixed: acinar (80%) and micropapillary (20%) | 2 | IA | 0 | 17 |
| 6 | 23 | Acinar | 1 | IA | 2 | 12 |
| 4 | 31 | Mixed: acinar (75%) and lepidic (25%) | 1 | IA | 1 | 10 |
| 17 | 29 | Mixed: papillary (70%) and lepidic (30%) | 1 | IA | 2 | 6 |
| 15 | 20 | Micropapillary | 2 | IA | 5 | 7 |
| 8 | 14 | Mixed: acinar (90%) and micropapillary (10%) | 2 | IB | 1 | 5 |
| 8 | 17 | Mixed: acinar (50%) and micropapillary (50%) | 2 | IIA | 4 | 1 |
| 22 | 30 | Misto: acinar (70%) and lepidic (30%) | 2 | IIA | 9 | 6 |
| 23 | 39 | Mixed: lepidic (80%) and micropapillary (20%) | 2 | IIA | 8 | 8 |
| 25 | 15 | Mixed: acinar (80%) and solid (20%) | 2 | IIA | 3 | 0 |
| 9 | 11 | Solid with mucus | 3 | IIA | 0 | 2 |
| 13 | 25 | Acinar | 2 | IIA | 4 | 1 |

*Table 10: Re-Tested wild-type patients stage III and IV: new mutation found with <5% allelic frequency by means of NGS*

| Patients ADK only | COVID-19 Affected Patients | Diagnosis (WHO 2015) | Grade | Stage | Mutated patients | Mutated patients COVID-19 |
|---|---|---|---|---|---|---|
| 8 | 35 | Solid with mucus | 3 | IIIA | 2 | 11 |
| 24 | 47 | Acinar | 3 | IIIA | 2 | 9 |
| 11 | 30 | Mucinous with lepidic mucinous fraction component | 2 | IIIA | 3 | 9 |
| 20 | 35 | Mixed: micropapillary (70%) and acinar (30%) | 2 | IIIA | 8 | 10 |
| 20 | 28 | Mixed: acinar (90%) and micropapillary (10%) | 3 | IIIA | 4 | 9 |
| 22 | 22 | Mixed: acinar (50%) and micropapillary (50%) with mucus and bezel ring cells | 2 | IIIB | 9 | 7 |
| 16 | 24 | Mixed: acinar (50%) and micropapillary (50%) | 3 | IIIB | 3 | 6 |
| 20 | 15 | Micropapillary | 3 | IIIB | 3 | 4 |
| 20 | 31 | Mucinous with lepidic mucinous fraction component | 3 | IIIB | 9 | 20 |
| 9 | 8 | Solid with mucus | 3 | IVA | 1 | 3 |
| 8 | 7 | Acinar | 3 | IVA | 3 | 2 |
| 9 | 17 | Mucinous with lepidic mucinous fraction component | 3 | IVA | 4 | 5 |
| 5 | 17 | Mixed: micropapillary (75%) and acinar (30%) | 3 | IVA | 0 | 3 |
| 17 | 34 | Mixed: acinar (80%) and micropapillary (25%) | 3 | IVB | 9 | 15 |
| 15 | 22 | Mixed: acinar (65%) and micropapillary (45%) with mucus and bezel ring cells | 3 | IVB | 2 | 10 |

Samples analysis carried out by NGS gave the possibility to find multiple mutations coexisting even at very low allelic frequencies (at least 1%). It was therefore possible to obtain a more specific molecular profile, both for the previously negative cases, and for those enrolled in 2021. Moreover, for cases (stage I and II), enrolled in the last year, were found mutated in 96/241 (39.8%) patients. (Table 11) On the other hand, considering results of the molecular biology of patients diagnosed lung ADKs with stage III and IV, showing invasive and metastatic histological characteristics, we report the results obtained in Table 12 from new cases enrolled during 2021 analyzed with NGS: multiple mutation found in 134/329 cases (40.7%). (Table 12)

*Table 11: New I and II stage enrolled patients'mutation status by means of NGS in 2021*

| Patients ADK only | COVID-19 Affected Patients | Diagnosis (WHO 2015) | Grade | Stage | Mutated patients | Mutated patients COVID-19 |
|---|---|---|---|---|---|---|
| 2 | 14 | Misto: acinar (60%) and lepidic (40%) | 2 | IA | 1 | 9 |
| 1 | 12 | Mixed: papillary (20%), lepidic (30%) and acinar (50%) | 1 | IA | 0 | 7 |
| 3 | 31 | Acinar | 2 | IA | 1 | 8 |
| 0 | 24 | Mixed: acinar (30%) and micropapillary (70%) | 2 | IA | 0 | 11 |
| 0 | 11 | Mixed: acinar (50%) and lepidic (50%) | 2 | IA | 0 | 6 |
| 1 | 29 | Mixed: papillary (60%) and lepidic (40%) | 1 | IA | 0 | 12 |
| 1 | 12 | Micropapillary | 2 | IA | 0 | 8 |
| 1 | 11 | Mixed: acinar (80%) and micropapillary (20%) | 2 | IB | 1 | 9 |
| 2 | 4 | Mixed: acinar (50%) and micropapillary (50%) | 1 | IIA | 0 | 2 |
| 3 | 8 | Misto: acinar (70%) and lepidic (30%) | 2 | IIA | 1 | 5 |
| 2 | 6 | Mixed: lepidic (70%) and micropapillary (30%) | 1 | IIA | 0 | 4 |
| 5 | 11 | Mixed: acinar (80%) and solid (20%) | 1 | IIA | 3 | 7 |
| 3 | 12 | Solid with mucus | 3 | IIA | 1 | 7 |
| 7 | 25 | Acinar | 2 | IIB | 4 | 10 |

*Table 12: New III and IV stage enrolled patients'mutation status by means of NGS in 2021*

| Patients ADK only | COVID-19 Affected Patients | Diagnosis (WHO 2015) | Grade | Stage | Mutated patients | Mutated patients COVID-19 |
|---|---|---|---|---|---|---|
| 2 | 11 | Solid with mucus | 2 | IIIA | 0 | 6 |
| 6 | 31 | Acinar | 3 | IIIA | 3 | 7 |
| 3 | 18 | Mucinous with lepidic mucinous fraction component | 2 | IIIA | 0 | 1 |
| 0 | 25 | Mixed: micropapillary (40%) and acinar (70%) | 2 | IIIA | 0 | 11 |
| 1 | 9 | Mixed: acinar (20%) and micropapillary (80%) | 3 | IIIA | 0 | 5 |
| 5 | 34 | Mixed: acinar (50%) and micropapillary (50%) with mucus and bezel ring cells | 2 | IIIB | 2 | 18 |
| 3 | 22 | Mixed: acinar (70%) and micropapillary (30%) | 2 | IIIB | 1 | 9 |
| 3 | 7 | Micropapillary | 3 | IIIB | 2 | 4 |
| 0 | 4 | Mucinous with lepidic mucinous fraction component | 3 | IIIB | 0 | 1 |
| 3 | 6 | Solid with mucus | 3 | IVA | 1 | 5 |
| 15 | 19 | Acinar | 3 | IVA | 6 | 11 |
| 4 | 7 | Mucinous with lepidic mucinous fraction component | 3 | IVA | 2 | 4 |
| 0 | 12 | Mixed: micropapillary (65%) and acinar (35%) | 3 | IVA | 0 | 7 |
| 3 | 11 | Mixed: acinar (50%) and micropapillary (50%) | 3 | IVB | 0 | 4 |
| 0 | 14 | Mixed: acinar (75%) and micropapillary (35%) with mucus and bezel ring cells | 3 | IVB | 0 | 9 |
| 0 | 16 | Mixed: acinar (60%) and micropapillary (40%) | 3 | IVB | 0 | 6 |
| 3 | 17 | Micropapillary | 3 | IVB | 0 | 5 |
| 1 | 14 | Mucinous with lepidic mucinous fraction component | 3 | IVB | 0 | 4 |

In most cases studied, especially using NGS method, multiple mutations have been found.

The fraction of patients who tested positive for most frequent mutational hot-spots and/or CNV in the ADKs population does agree with what is reported in the main scientific journals.[137]

Below is reported a complete list of the total mutations that have been detected with both MS and NGS methods.(Table 13)

ADK cases tested by means of MS, found negative, and re-tested with NGS are a total of 715/2017 (35.4%); they presented driver mutations and/or simultaneously non-targetable hot-spot and/or

mutations and/or CNV. Morover, the same percentage of mutation has been found among ADKs stage I and II; and among ADKs stage III and IV.

In table 11, all hot-spot mutations and CNV found in different genes have been reported in detail.

*Table 13: Mutation detected with both MS and NGS*

| Gene | Mutation | Mutations number | Gene | Mutation | Mutations number | Gene | Mutation | Mutations number | Gene | Mutation | Mutations number |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***ALK*** | C1116Y | 8 | ***KRAS*** | G12D | 71 | ***EGFR*** | V774_C775insHV | 2 | ***TP53*** | R213Q | 2 |
| ***ARAF*** | S214F | 1 | ***KRAS*** | G12F | 6 | ***EGFR*** | L747_A750>P | 12 | ***TP53*** | R249M | 1 |
| ***BRAF*** | V600E | 142 | ***KRAS*** | G12V | 94 | ***EGFR*** | L747_A753>S | 6 | ***TP53*** | R249S | 1 |
| ***BRAF*** | D549G | 2 | ***KRAS*** | G12R | 7 | ***EGFR*** | L747_A753>Q | 1 | ***TP53*** | R282K | 1 |
| ***BRAF*** | D549A | 2 | ***KRAS*** | G12S | 7 | ***EGFR*** | L747_T751delLREAT | 23 | ***TP53*** | R283P | 1 |
| ***BRAF*** | G469A | 7 | ***KRAS*** | G12L | 2 | ***EGFR*** | L747_T752delLREATS | 15 | ***TP53*** | Q192* | 1 |
| ***BRAF*** | G469E | 1 | ***KRAS*** | G13D | 20 | ***EGFR*** | H773_V774insH | 2 | ***TP53*** | R248Q | 2 |
| ***BRAF*** | G469V | 2 | ***KRAS*** | G13C | 17 | ***EGFR*** | H773_V774insNPH | 1 | ***TP53*** | R273H | 1 |
| ***BRAF*** | G466A | 1 | ***KRAS*** | G13V | 1 | ***EGFR*** | S752_I759delSPANKEI | 17 | ***TP53*** | S241F | 1 |
| ***BRAF*** | G466V | 2 | ***KRAS*** | Q61K | 3 | ***EGFR*** | Del19 generic | 72 | ***TP53*** | R248L | 1 |
| ***ERBB2*** | Y772_A775dup | 2 | ***KRAS*** | Q61L | 5 | ***EGFR*** | Ins20 generic | 23 | ***TP53*** | H179Y | 1 |
| ***ERBB3*** | V104L | 1 | ***KRAS*** | Q61H | 14 | ***EGFR*** | E709D | 1 | ***TP53*** | H179R | 1 |
| ***EGFR*** | G719A | 20 | ***KRAS*** | Q61R | 2 | ***EGFR*** | E709K | 1 | ***TP53*** | R237S | 1 |
| ***EGFR*** | G719C | 2 | ***NRAS*** | G12V | 12 | ***EGFR*** | L747P | 4 | ***TP53*** | V15F | 1 |
| ***EGFR*** | G719X | 6 | ***NRAS*** | Q61L | 6 | ***EGFR*** | L858R | 174 | ***TP53*** | L194R | 1 |
| ***EGFR*** | D770_N771insSVD | 2 | ***NRAS*** | Q61R | 11 | ***EGFR*** | L861Q | 7 | ***TP53*** | G262V | 1 |
| ***EGFR*** | D770_N771insGF | 1 | ***NRAS*** | Q61H | 5 | ***EGFR*** | S768I | 7 | ***TP53*** | G245V | 1 |
| ***EGFR*** | N771_P772insTP | 2 | ***NRAS*** | Q61K | 7 | ***EGFR*** | T790M | 83 | | | |
| ***EGFR*** | E746_A750delELREA | 54 | ***MET*** | D1028H | 1 | ***EGFR*** | L833V | 1 | **Gene** | **CNV Type** | **CNV number** |
| ***EGFR*** | E746_A750fs* | 6 | ***MET*** | R988C | 1 | ***FGFR3*** | F348R | 1 | ***CDKN2A*** | Loss | 10 |
| ***EGFR*** | E747_A750>P | 12 | ***PIK3CA*** | E545K | 13 | ***HER2*** | E770_A771>AYVM | 1 | ***ERBB2*** | Amplification | 1 |
| ***EGFR*** | E746_S752 | 11 | ***PIK3CA*** | E542K | 16 | ***HER2*** | A775_G776>YVMA | 1 | ***FGFR1*** | Gain | 1 |
| ***EGFR*** | E747_S752 | 9 | ***PIK3CA*** | H1047R | 3 | ***HER2*** | A775_G776>IVMA | 1 | ***FGFR2*** | Gain | 1 |
| ***EGFR*** | E746_S752>V | 20 | ***PIK3CA*** | G1049R | 2 | ***KRAS*** | G12A | 31 | ***FGFR3*** | Gain | 2 |
| ***EGFR*** | A767_V769dup | 2 | ***PIK3CA*** | C420R | 1 | ***KRAS*** | G12C | 167 | ***KRAS*** | Amplification | 2 |
| ***EGFR*** | V769_D770insASV | 2 | ***TP53*** | R175H | 1 | | | | | | |

The most recurrent driver mutations in our series mirror those that have always emerged in the mutation studies of ADKs. Particular relevance are mutations in *EGFR* exon 19, in which we found the presence of deletions in 259/2017 (12.8%) cases. It was not always possible to resolve the extent of some deletion depending on the method used and the amount of DNA available.

In *EGFR* exon 20 we found the presence of the sensitivity mutation p.L858R in 174/2017 (8.6%) of patients. Resistence mutation (i.e.: p.L747P in exon 19, Ins exon20 and p.T790M) have been found in 123/2017 (6%), even coexisting with sensitive mutations.

A good fraction of cases 447/2017 (22%) showed the mutation for *KRAS* gene, in exon 2 (codon 12 or 13) or for exon 3 (codon 61). Mutation rate for all other genes that were detected was 13.5% (273/2017). Some of these mutations are not always subject to personalized therapy,

however, it is important to know their existence with the role of accompanying mutations; some of them have a prognostic value.

Although 71% of patients were affected by COVID-19, in addition to ADKs, results obtained are in line with those that we normally obtained from a population affected by lung cancer. Mutation frequencies, ADKs histotype, stage and grade, do not seem to correlate with COVID-19 infection, which seems to have an independent evolution from neoplasm. Specifically, the portion of our cohort classified as early stage, driver mutations of lung disease have the same incidence that is normally found in lung tumors. These results not only correlate between ADKs-COVID-19 and -COVID-free patients, but also correlate with literature data.[137,138]

### *EasyPGX® ALK\ROS\MET\RET*

Good quality RNA was extracted from FFPE samples examinated.

Among cases studied, starting material was bone marrow biopsy for 15 patients (8 ADKs-COVID-19 free, 7 ADKs-COVID-19).

Using a qualitative method, it was only possible to determine the presence/absence of a rearrangement, at level of specific regions involving translocations of target genes involved in ADKs and tagetable with approved molecular therapies. We found the presence of rearrangement for *ALK* gene in 9 cases in which the imbalance between 3' and 5' portion of gene has been detected. *ROS1* was found translocated in 3 cases: 1 case tested positive for the rearrangement involving exon 32, while, other 2 cases presented rearrangement involving exon 34. 5 cases found to be rearranged for the *RET* gene, were all related to the exon 12.

Moreover, 8 patients were positive for *cMET* exon 14 skipping mutations, without having the specific nucleotide/protein of the mutation.

Bone marrow tissues, as per standard anatomic pathology procedures, are subjected to decalcification protocols in order to be subsequently cut with microtome. Therefore real-time investigations failed; an attempt was to analyze them with alternative IHC and FISH methods.

## *4.3.3 Immunohistochemistry and Fluorescence in situ hybridization*

Not for all samples it was possible to proceed with the real-time PCR investigations to detect *ALK, ROS1* and *RET* gene rearrangements: in addition to the quality of the RNA, the starting biological material quantity is also important. For many cases, biopsy samples were poor, rare and/or with exiguous neoplastic cellularity, borderline with adequacy cut-off. Therefore, methods such as IHC and FISH were carried out to preserve the biological material and try to

produce diagnostic results for setting up personalized therapy. IHC and FISH methods were found to be useful for all those for which their processing had to have particular attention. However, we found the presence of translocations involving *ALK* gene in 63 cases (27 ADKs-COVID-19 free, 36 ADKs-COVID-19. Data obtained from both FISH or IHC; sometimes from both methods to confirm the result); 21 cases resulted positive for the *ROS1* gene, while 28 case, of which 5 presented both overexpression and translocation of *RET* gene: mean >5 signals/nucleus and co-presence of the translocation in more than 30% of nuclei. These are partial data obtained from IHC and FISH. Even these resuts do not correlate between ADKs-COVID-19 and -COVID-free patients, as describe before in mutational analyses pharagraph. Biological starting material, in patients with COVID-19, was pretreated more aggressively due to microcalcifications and fibrosis present in the tissue.(Images IHC 6, 7 and 8; FISH 9, 10, 11 and 12)

### *4.3.4 cMET exon 14 skipping mutations*

Amplifiable DNA was extracted from tested material. These are mainly small biopsies in which the amount of DNA was smaller and, for DNA extracted from patients with COVID-19, as previously described, the nucleic acid was found to be of a lower quantity but qualitatively sufficient for amplification. From all cases studied it was possible to obtain amplicons up to about 250bp from three couple of primers that mapp the region between *cMET* intron 13, exon 14 and intron 14. *cMET* exon 14 skipping mutations has been evaluated in all cases who there was poor quality and quantity of starting FFPE tissue. *cMET* gene skipping mutations, as per diagnostic protocols, were evaluated in all cases negative for *EGFR* gene. There have been several cases with low quantity and quality of starting material, so we proceeded to verify the presence of these mutations by means of Sanger method.

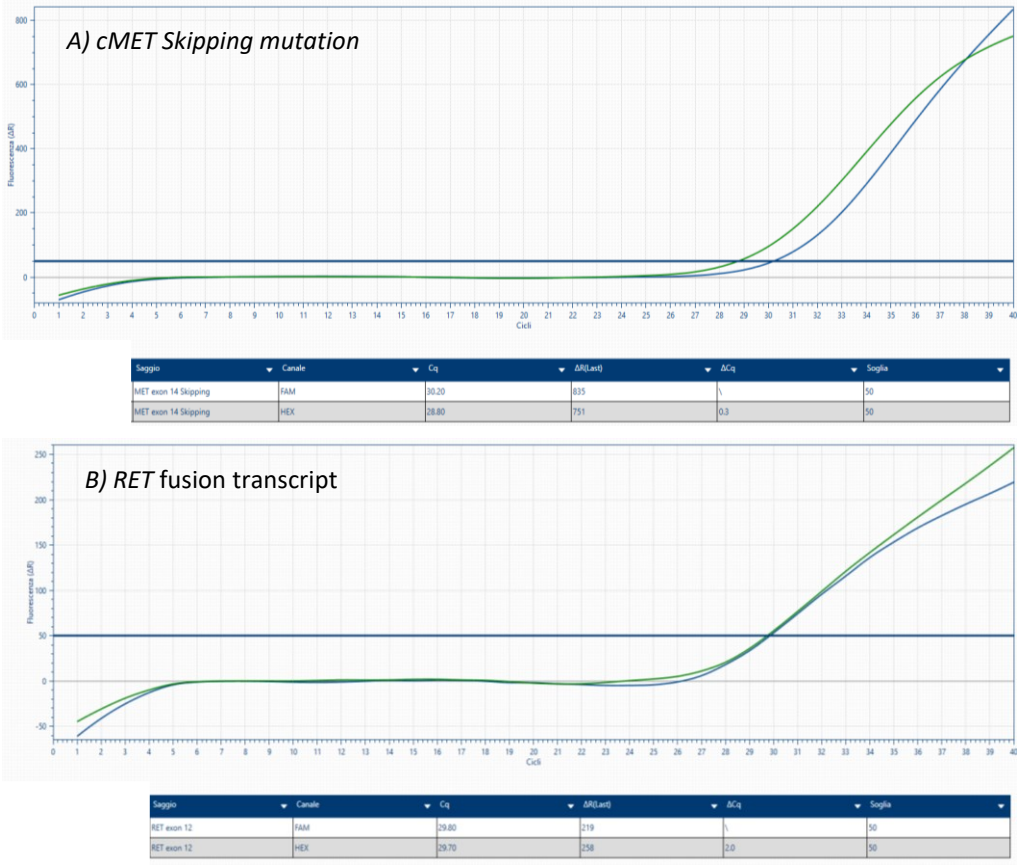
Using Sanger sequencing, it was possible to find out 16 (6 ADKs-COVID-19 free, 10 ADKs-COVID-19) patients with skipping mutation that are listed below (Table 14):

*Table 14: Exon 14 Skipping mutations details*

| Patients number | *cMET* Exon 14 alteration (gDNA;cDNA) | Type Alteration | Sanger Sequecing inerpretation |
|---|---|---|---|
| **8** | g.116412044G>T; c.3028+1G>T | base substitution splice donor | Substitution |
| **4** | g.116412043G>A; c.3028G>A | base substitution splice donor | Substitution |
| **1** | g.116412033_116412045del13; c.3018_3028+2delTTTTCCAGAAGGT | Indel splice donor | Deletion |
| **2** | g.116411894_116411896delinsGG; c.2288-9_2288-7delinsGG | Indel/base substitution splice acceptor | Deletion |
| **1** | g.116411748_116412484del737; c.2287+40_3028+441del | whole exon 14 deletion | wild type |

Considering different methods used for the study of the *ALK*, *ROS1*, *RET* and *cMET* genes, which are necessary to provide personalized therapy, we can define that even in our cohort, these mutations, unlike other drivers and targetable ones, are present with much less frequency, as normally described in literature. In particular, *ALK* (3%), *ROS1* (1%), *RET* (2%), finally *cMET* (3%).(Figure 5)

*Figure 5: A) cMET skipping mutation exon 14; B) RET fusion rearrengement exon 12*



### *4.3.5 Liquid Biopsy*

1516 liquid biopsies have been evalutated with Easy®EGFR (Diatechpharmacogenetics, Jesi, Italy).

The kit contains main mutation in *EGFR* exons 18, 19, 20 and 21 by means of 8 master mix of oligonucleotides. It was chosen to use this type of investigation for two main reasons: 1) to look for disease-driving mutations at time zero in non-biopsiable patients; 2) monitor in predetermined times, and if the general conditions of the patients under examination could consent, the possible progression or stabilization of the disease. Given the rarity of the circulating tumor cells, and therefore of the ctDNA that can be eluted, the use of a kit with few but specific mutations has been established, aimed above all, at monitoring the progression of the disease with the appearance of the p.T790M mutation. 223/1516 liquid biopsies (14.7%), were positive for mutation. In particular 1.3% (indel exon 20) and 2.1% (p.T790M): drug resistance mutations for TKIs first-generation treatments; 5.1% (exon 18 point mutations), 54.9% (exon 19 deletions), 5.1% (exon 20 point mutations), finally 31.5% (exon 21 point mutations): mutations that confer sensitivity to treatment with TKIs drugs.(Table 15) (Figure 6)

Many of the mutations found in liquid biopsy correspond to those previously analyzed of the corresponding biopsy specimens/pieces or creators. The patients who tested positive for *EGFR* gene (by means of MS and/or NGS), were further tested, in pre-established timings to verify lung disease progression and, above all, the possible presence of resistance mutations.

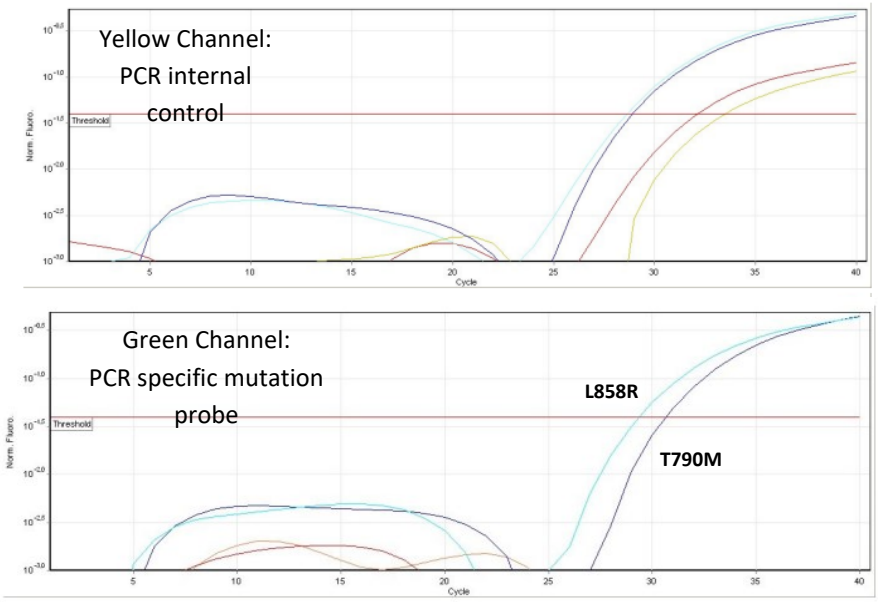
*Table 15: Liquid Biopsies sensitive and resistence mutations detected*

| Exon | Sensitive Mutation | Frequency |
|---|---|---|
| Exon 18 | c.2155G>A (p.G719S)<br>c.2155G>T (p.G719C)<br>c.2156G>C (p.G719A)<br>c.2159C>T (p.S720F) | 5,1% |
| Exon 19 | deletion exon 19 | 54,9% |
| Exon 20 | c.2294C>T (p.V765A)<br>c.2347A>C (p.T783A)<br>c.2143G>T (p.S768I) | 5,1% |
| Exon 21 | c.2573T>G (p.L858R)<br>c.2582T>A (p.L861Q)<br>c.2582T>G (p.L861R) | 31,5% |

| Exon | Resistance Mutation | Frequency |
|---|---|---|
| Exon 19 | c.2281G>T (p.L761Y) | |
| Exon 20 | D770_N771 (p.insNPG)<br>D770_N771 (p.insSVQ)<br>D770_N771 (p.insG) | 1,3% |
| Exon 20 | c.2369C>T (p.T790M)<br>c.2395G>T (p.V765L)<br>c.2312A>C (p.N771T) | 2,1% |

The 18 cases (5 ADKs-COVID-19 free, 13 ADKs-COVID-19) of patients with ADK, of which it was not possible to take any biopsy due to the advanced stage of the disease, rather than for the unreachability of the tumor site, it was possible to evaluate the presence of mutations in *EGFR* gene in 4/18 cases (22%), a significant data if we consider all technical difficulties related to the isolation of the ctDNA and the RT-PCR test mutation sensitivity with 3-5% (average of mutation-specificity).

*Figure 6: RT-PCR qualitative assay. A) Amplification internal control for each sample tested; B) Patient with driver primary mutation p.L858R and mutation novel resistance p.T790M.*



# 5. DISCUSSION

In this study, we used different diagnostic methods to analyze patients affected both by ADKs-COVID-19 free and ADKs-COVID19 (71% of the entire cohort), from a molecular and genetic point of view. These results were then compared with the patient's histological grade, stage and state of health relative to the viral infection (data not shown: data relating to patient privacy).

It should be specified that, starting from "Time 0", about 23% of the cases among the patients enrolled as ADKs-COVID-19 free, showed the viral infection during the two years of the pandemic.

The series included 2017 patients who previously underwent triage and CT procedures, than needle aspiration biopsy (total collected 2473), and/or subsequently lobectomy (total collected 776).

18 patients were immediately excluded from the study as regards any type of tissue sampling; for they there was possible only proceed by means of liquid biopsy. Also all the other patients, if it was possible and compatible with the patients' compliance and health *status*, underwent blood samples for liquid biopsy (total collected 1516) as a follow-up.

Samples were received, analyzed and characterized at the Unit of Anatomic Pathology IRCCS Humanitas Clinical Reasearch Institute, during the pandemic, from March 2020 to December 2021.

The multidisciplinary team is still joining forces to give patients the best possible cares.

Our study allowed us to identify the presence of driving mutations through MS and/or NGS methods in 35.4% of cases.

Although specifically, MS has a 5% of sensitivity, as compared to a Real-Time PCR and NGS (with higher sensitivity 1-2%). Furthermore, despite that the NGS System used, is not yet equipped with validation at European level for diagnostics (IVD-CE marked), we have tested so many cases as we can claim to perform an internal validation.

Specifically, a total of 715/2017 (35.4%) ADKs resulted mutated after been tested by means of MS, and re-tested with NGS; they presented driver mutations and/or simultaneously non-targetable hot-spot and/or mutations and/or CNV, as reported in Table 13.

We did not distinguish between mutations, histological stage and grade, and presence/absence of COVID-19 viral infection, because the neoplastic events were identified as independent.

Morover, the same percentage of mutation has been found among ADKs stage I and II; and among ADKs stage III and IV. These percentages, not very different from each other, confirm how many driver mutations can arise early stages with low allelic frequencies (even <10%); as far as showing high allelic frequencies (over >50%) in the advanced stages of the disease. In fact, these patients, if positive for targetable mutations, were eligible to personalized treatment. As regards ADKs early stage patients, it was possible, always compatibly with the viral infection, to proceed with the

surgical resection and prevent the develop of aggressive lung neoplasm. To date, these patients, who have also recovered and survived from the viral infection, have recurrence of the disease in only 2%. For other patients with stage III and IV, althought physical conditions of many of them were not optimal due to the COVID-19 viral infection, the diagnostic imaging (CT-scan) showed a regression of tumor size and metastases, in response to drugs, regardless of COVID-19. The survival of these patients, as previously mentioned, was however shorter due to the onset of pulmonary insufficiency, embolism and thrombosis due to the virus.

Although about 71% of patients were affected by COVID-19, in addition to ADKs, results obtained are in line with those that are normally obtained from a population affected by lung cancer. Mutation frequencies, histotype, stage and grade, do not seem to correlate with COVID-19 infection, which seems to have an independent evolution from neoplasm. Specifically, also with regard to the portion of the cohort classified as early stage, driver mutations of lung disease have the same incidence that is normally found in lung tumors. These results not only correlate between ADKs-COVID-19 and -COVID-free patients, but also correlate with literature data.[137]

For this reason the mutational results were tabulated without differentiating them between histological subgroups, stage, grade and related COVID-19 infection. Furthermore, the collection of some of these data was rather rapid and without follow-up: it was inevitable because of the aggressive trend of COVID-19 who did patients not to survive.

Although the data obtained are good, some discrepancies were nevertheless found; discrepancies between results obtained from biopsy *versus* correspondent surgery sample (3%), collected in different timing, can be explained: it is possible that, as regarding biopsy, at the time of DNA/RNA isolation, the amount of neoplastic cells remaining in the paraffin block was much less than the amount needed for assays. For this reason, results obtained did not agree with the molecular-genetic ones starting from surgery FFPE samples.

Patients mutated in *EGFR* gene, and for the 18 cases that could neither be biopsied nor underwent suergery, were subjected to evaluation with the Real-Time PCR method to detect the presence of a driver mutation detectable through this method (in 4/18 of cases). The liquid biopsy sampling was also used to monitor patients with TKIs sentitive *EGFR* mutations (detected by means of MS and/or NGS methods) in probable disease progression: the evaluation of resistance mutations has made it possible to reset therapy with specific second-generation TKIs.

As regards these investigations it was not always possible to verify the presence of a pre-existing and already identified mutation; the method presents pitfalls that concerns the quantity of verifiable mutations, albeit with a rather good sensitivity and specificity, but above all the greatest limit

derives from the starting material: circulating tumor cells and the quantity of ctDNA that is possible to isolate.

As previously explained, gene translocation and *cMET* exon 14 skipping analyzes were carried out with a second method for all those samples with not optimal biological material: IHC assays (ALK protein expression), FISH (*ALK*, *ROS1* and *RET* genes) and Sanger sequencing for *cMET* gene.

The identification of translocations involving *ALK*, *ROS1*, *RET* and *cMET* (exon 14 skipping mutations) genes by RT-PCR in our cohort, revealed the presence of genetic aberrations in: 3% for *ALK*, 1% for *ROS1*, 2% for *RET* gene and 3% of *MET* exon 14 skipping mutations.

As regards *cMET* gene, all those cases that did not showed any mutation for *EGFR* were studied, as required by diagnostic steps for the therapeutic setting. Therefore, given that *EGFR* was mutated in 29.7% of cases (601/2017 patients in total); remaining cases were tested for *cMET* gene. As regards data obtained from our investigations, are in agreement with what have been reported in the literature.[139,140]

Even from results obtained with IHC and FISH methods, a mismatch was found in obtained data between biopsy and corresponding surgery sample (5% discordance data); this could be attributed to intratumoral heterogeneity of tumour cells; from a quantitatively limited material such as the bioptic one.

This is what we can also observe thanks to clinical evidence (data not shown: data relating to patient privacy). Treatment of ADKs has greatly improved over the past few decades thanks to identification and introduction into clinical practice of new active drugs and the combination of various therapeutic modalities. In particular, the integration of surgery, radiotherapy, chemotherapy, the introduction of molecularly targeted therapies and the more recent advent of immunotherapy have radically changed the therapeutic algorithm. Mutations in *EGFR* gene in patients with NSCLC are associated with sensitivity for treatment with TKIs, as previousely described.

From clinical data collected during the first year of study: patients affected of ADKs early stage, were mostly followed-up or treated with low-dose first-generation TKI after surgery; some of them presented a slower disease relapse than a patient diagnosed at stage III-IV.

Up to date, only 2% of patients who have been diagnosed at stage I and II, are in progression of the disease. For all other cancer stages Clinicians were able to use both imaging (TC-scan), tissue rebiopsy and/or liquid biopsy to monitor the progression of the disease and reset therapy.

Although studies have been published encouraging results in terms of progression free survival (PFS) for the use of these drugs as a first line of treatment, many patients develop resistance to therapy after the first 8-16 months, like in our cohort.[123,126] In particular, the p.T790M mutation (exon 20) appears to be the most frequent *EGFR*-TKIs-resistant mutation, with an incidence of 60-

70%. Recently, *EGFR*-TKIs molecules have been developed that target the p.T790M mutation, overcoming the obstacle of drug resistance and showing positive results for both PFS and ORR in patients who have had disease progression after first-generation TKIs treatment. To date, there are two target drugs for the p.T790M mutation: Osimertinib and Rociletinib, which are used as new second-generation treatments in ADKs.[142] Some of patients developped the presence of p.T790M resistance mutation after one year.

To date, clinical studies allow the treatment of other mutations that may arise in patients with ADKs, including *BRAF* p.V600E (Dabrafenib combined with Trametinib in metastatic ADKs), already among the first mutations to be targeted and finally approved by Agenzia Italiana del Farmaco (AIFA) in 2020.[143]

Moreover, many of our patients with mutation in *KRAS* exon 2, p.G12C, were treated with Sotorasib, the new III phase trial has been activated in May 2021 by FDA approved as the first treatment for adult patients with NSCLCs. The major outcomes measured were objective response rate (proportion of patients whose tumor is destroyed or reduced) and duration of response. We are still collecting data to be able to compare them with those described in the literature: the objective response rate (ORR) for patients treated with *KRAS* inhibitor was 36% and 58% of those patients had a duration of response of six months or longer.[144] These just described are average percentages in response to drug therapy, considering that most of patients of our cohort were also affected by COVID-19, our ORR will certainly be lower. These patients, where possible, followed the same therapies like patients with ADKs-COVID-19 free, receiving at the same time anti-viral drugs.

Results we obtained for the research of the translocations of the *ALK* gene do not seem to confirm a correlation between age and smoking habits: almost 456 patients of our series were older than 60 years and some of them had a smoker history (data not shown in tabels. These data derive from the anamnestic information present in patients'medical records that cannot be published in this thesis work). Focusing on *ALK* gene translocations, targetable with Crizotinib, like *ROS1* gene, many studies have reported a variedly documented association between a solid-type histological pattern with signet ring cells [relatively rare in the different histological patterns of lung ADKs[1]] and the translocations of the *ALK* gene.[145,146-149] However translocations have also been documented in ADKs with more conventional histology, of the papillary and acinar type [4/29 in the McLeer series[150]], or colloids with abundant extracellular mucus [2/4 in the Just 2011 series[151], 56% of the cases described by Yoshida 2011[152]].

Although the search for translocations involving *ALK* and *ROS1* genes, through the use of break-apart type probes for FISH investigations, is currently considered the gold standard for the selection of patients to be treated with Crizotinib, this method is relatively expensive, time-consuming and

above all it must be interpreted by experienced and qualified staff. This is in fact a focal point for the interpretation of FISH break-apart probes rearrangements in general, but most of all for *RET* gene. In our cohort we were able to validate some anomalous *RET* patterns found by FISH with at least two methods, but not all of them were confirmed by RT-PCR. Recombinant *RET* patterns evaluated in FISH do not always give a functional rearrangement, therefore, personalized therapy with Vandetanib would be useless.[153]

It would be desirable to have the possibility of verifying *RET* functional rearrangements by means of NGS or RT-PCR panels. Our group is dealing with this through the NGS tool in use. We are still in validation phase; but to date, although the number of cases tasted is not yet as numerous as for the DNA analysis we presented in this large study, we know we have a rather powerful instruments, that will allow us to analyze gene rearrangements and, possibly, to delete personal interpretation bias that arises in reading FISH samples (concordance percentage between methods in our internal validation is 90%).

A problem of great importance is therefore study the molecular profile on the scarce material obtained with a biopsy sample; if the sample is sufficiently representative of the molecular structure of the neoplasm in its entirety and can justify the demanding therapeutic choices both in terms of side effects that cost.

# 6. CONCLUSION

SARS-CoV-2 pandemic has severely affected patients with NSCLCs and impaired the progress of lung cancer research. Patients with lung cancer are at an increased risk of becoming infected with the virus and experience higher morbidity and mortality than the general population.[49]

At the beginning of the pandemic, patients with lung cancer were deprioritized for the admission or mechanical ventilation in intensive care units, with an increasing rate of death.

To date, based on the acquired knowledge on the impact of COVID-19 in patients with cancer, this approach appears out of order, confirming that patients with lung cancer should not be excluded from intense care units and should be prioritized to receive a prompt and comprehensive care, as other patients.

Despite challenges posed by the pandemic, there has been an unprecedented international effort to understand the impact of COVID-19 on this patient population. Cancer societies, including ESMO and ASCO, have released statements and guidelines to help clinicians manage patients with lung cancer during this health crisis.[68,88] It is for this scenario of clinical management that the need arose in our Institute to create a multidisciplinary group that would monitor and maintain constant care for cancer patients, especially, as we have said, for NSCLC. Based on clinical studies and guidelines that were gradually outlined, we have created, to date, a network that seems to work fluently, despite COVID-19 infection, still lethally affecting many of these patients, who are part of the so-called fragile population. Goal of our work is to improve outcomes in patients with lung cancer, especially ADKs, infected with the virus.

Studies such as TERAVOLT and CCC19 have provided essential information on the effect of different treatment modalities on patients with lung cancer in the context of COVID-19.[51,60]

Regarding the clinical research, FDA and EMA have also released guidelines for continuing clinical research during the pandemic.

As the number of COVID-19 cases continues to surge across the globe, it is becoming increasingly evident that the incidence of COVID-19 in patients with lung cancer will continue to rise, at least until populations are effectively vaccinated.[65]

Even in our cohort, patients underwent vaccination coverage as soon as inoculation was possible. Only a small fraction of the patients in our study, about 10%, were able to develop immunity to COVID-19 thanks to vaccines (up today are still alive). Despite vaccination coverage, many patients with severe health conditions fell ill a second time for COVID-19 and did not survive. Moreover, unfortunately, chemotherapy drugs have created deficits in the immune system (data not shown: data relating to patient privacy). Further research and close monitoring of patient clinical

outcome and progress, needed to understand the mechanisms that lead to increased susceptibility and mortality in patients with ADKs infected with SARS-CoV- 2.

As previousely annouced, all clinical data about terapeutic, prognostic and outcome of patient, as well as the immunological *status* (related to the vaccine), will be collected and better discussed at the end of the study estabilished by June 2022 and discussed in a separated clinical study.

To date, as supported by the main international worldwide oncology societies, including American Association for Cancer Research (AACR),[154] ASCO,[155,156] ESMO,[157] National Comprehensive Cancer Network (NCCN) and Society for Immunotherapy of Cancer (SITC),[158] patients with cancer and in particular lung cancer should receive an approved COVID-19 vaccination, also for those undergoing treatments, including chemotherapy, target agents and immunotherapy. Furthermore, as the development of new therapies to treat or prevent COVID-19 infection increases, evaluating the efficacy and side effects of these therapies, and their interaction with anticancer agents including effects of the vaccines will be essential for continuing to care for patients with NSCLCs.

The COVID-19 pandemic has affected the care of patients with lung cancer and has affected multiple aspects of cancer research.[90] Since the beginning of the pandemic, there has been a decrease in clinical trial enrollment, and several active trials have been placed on hold.[91]

While data demonstrating the effect of COVID-19 specifically on lung cancer research is rare, several studies show that cancer research in general has been compromised.[92-98]

Despite this situation, our Center has set up an internal task force, dealing with the diagnostic guidelines that were gradually updated during the pandemic, to treat ADKs-COVID-19 and ADKs-COVID-19 free patients in the best possible way. The pandemic has made us possible to identify a higher fraction of patients with ADKs than what normally happens. Moreover it has been possible to isolate many more lung tumor ealy stage, resectable and treatable tumors.

With our study we would show that using a structured method and relying on a diverse panel of experts, we have developed a detailed set of clinical statements to guide health care professionals and assist patients in many of the clinical and technical obstacles related to diagnosis, risk assessment, response assessment, surgical planning, RT and personalized treatment during the COVID-19 pandemic.

Although about 71% of patients were affected by COVID-19, in addition to ADKs, results obtained are in line with those that are normally shown from a population affected by lung cancer. Mutation frequencies, ADKs histotype, subtypes and grade, do not seem to correlate with COVID-19 infection, which seems to have an independent evolution from neoplasm.

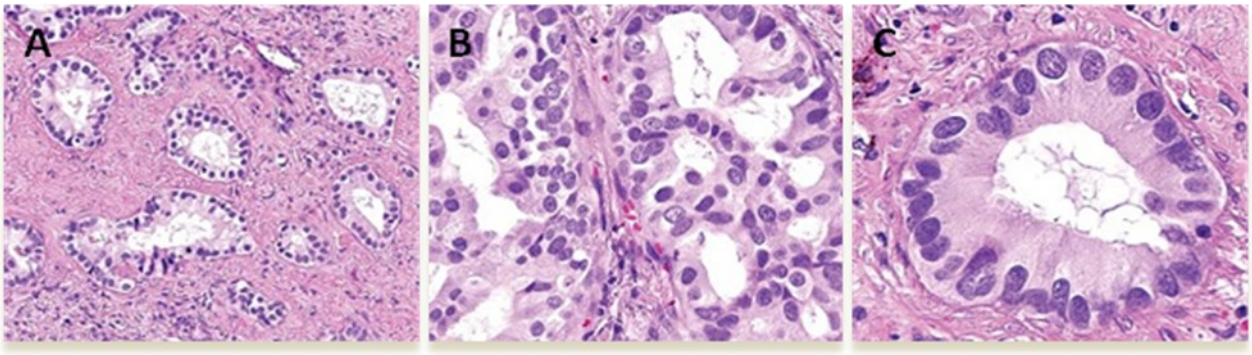
Specifically, also with regard to the portion of the cohort classified as early stage, driver mutations of lung disease have the same incidence that is normally found in lung tumors. These results not only correlate between ADKs-COVID-19 and -COVID-free patients, but also correlate with literature data. For this reason the results were tabulated without differentiating them between histological subgroups, grade and related COVID-19 infection.

The difficult management of cancer patients, simultaneously affected by COVID-19 during the pandemic has given us the opportunity to verify the feasibility of oncological treatments, any bias that could have occurred on the evaluation of driver mutations and on the response to target therapies. Despite the high mortality rate (from 35 up to 40% of patients worsened and died due to viral infection during these two years), we were able to verify that there is no correlation between COVID-19, the progression of neoplastic progression or the generation of mutations that can induce lung cancer. The response to target therapies also had the desired effects, albeit more slowly, given the suffering conditions of the lungs. From this work, which is still in the progress and implementation phase, we have therefore defined that the lung neoplastic pathology of ADKs (and everything that follows it, from diagnosis to therapy), proceeds independently from COVID-19 infection.

Ultimately, in the last two years we aquired repository knowledge that will be better informed by accumulating data on SARS-CoV-2, its pandemic characteristics, risks, and its modulating factors in cancer patients and, finally, try to guarantee an optimal cancer care in presence of the virus.

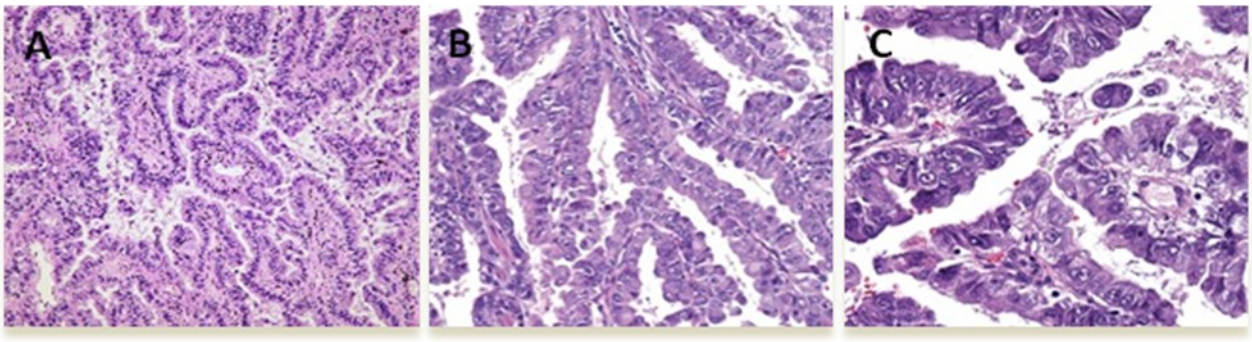
# 7. ICONOGRAPHIC SECTION

**Image 1:** ADK examples – acinar subtype



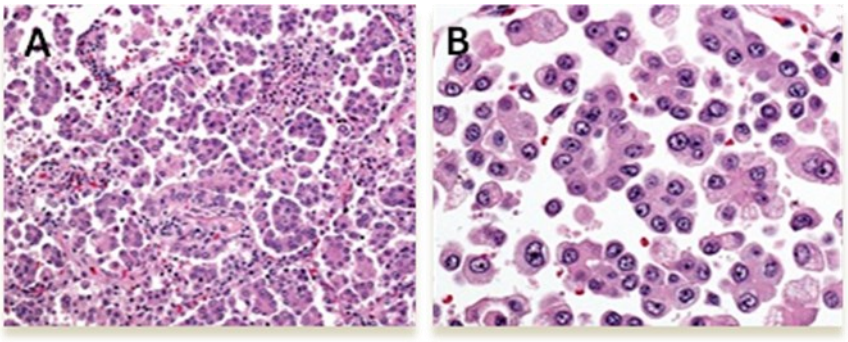

**A:** cellular structures organize themselves forming pseudo-glandular aggregates. The cells appear clear due to the considerable presence of mucus (Hematoxylin-Eosin, 10X). **B, C:** detail of a glandular formation; inside it is clearly visible the accumulation of mucin (Hematoxylin-Eosin, 40X).

**Image 2:** ADK examples papillary subtype



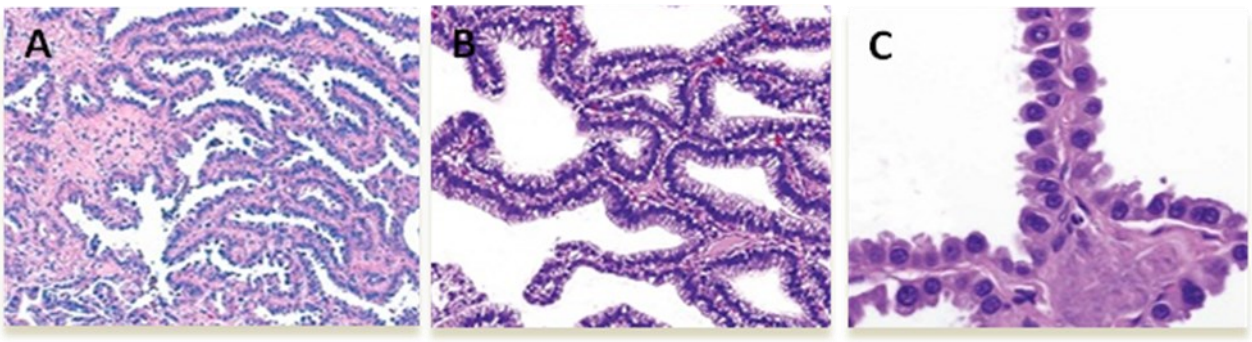

**A:** characterized by papillae with secondary and tertiary papillary structures that replace the pulmonary architecture that surrounds them (Hematoxylin-Eosin, 10X). **B:** detail of a branched papilla (Hematoxylin-Eosin, 20X). **C:** detail of an unbranched papilla (Hematoxylin-Eosin, 40X).

**Image 3:** ADK examples micropapillary subtype



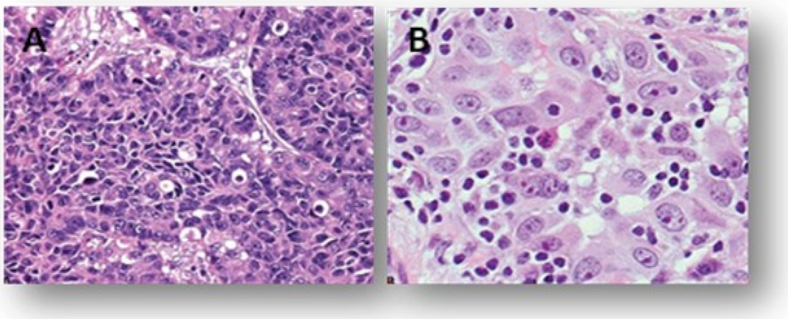

**A:** cellular architecture characterized by the lack of a central fibrovascular nucleus (Hematoxylin-Eosin, 10X). **B:** detail of micropapils (Hematoxylin-Eosin, 20X).

**Image 4:** ADK examples lepidic subtype



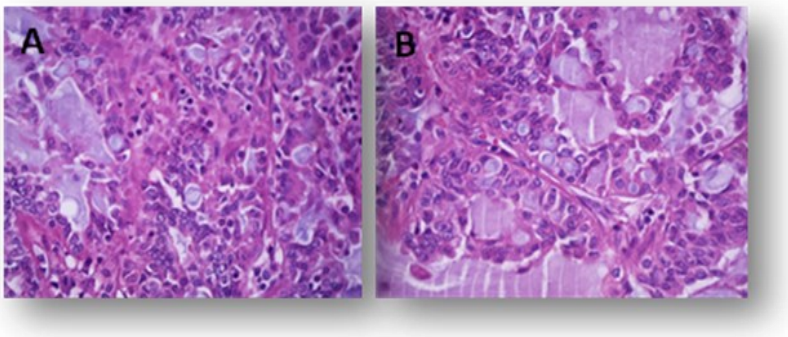

**A and B:** the growth of the neoplastic cells along the pre-existing alveolar structures can be observed, without stromal evidence (Hematoxylin-Eosin, 10X). **C:** detail of lepidic architecture, neoplastic cellular monolayer that covers the wall of the alveoli (Hematoxylin-Eosin, 40X).

**Image 5:** ADK examples solid subtype



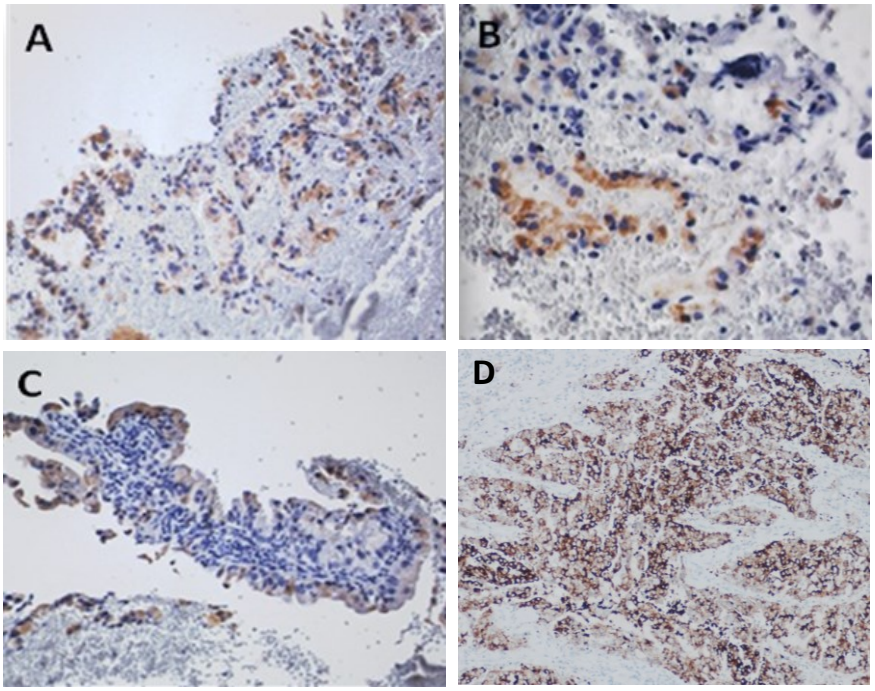

**A:** this architecture is characterized by very close groups of neoplastic cells, which lack acinar, tubular or papillary architecture (Hematoxylin-Eosin, 20X); **B:** detail (Hematoxylin-Eosin, 40X).

**Image 6:** ADK examples mucinous with goblet cells




**A, B:** Hematoxylin-Eosin (40X)

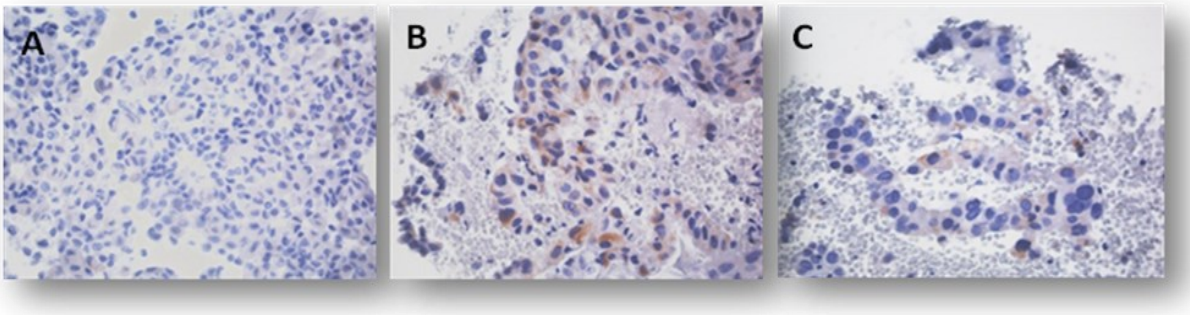
**Image 7:** ADK, IHC ALK on tissue biopsies (A,B,C) and Surgery sample (D)




**A** (10X), **B** (20X) and **C** (10X) the cytoplasm of almost all cancer cells has an intense mark (score 2+).

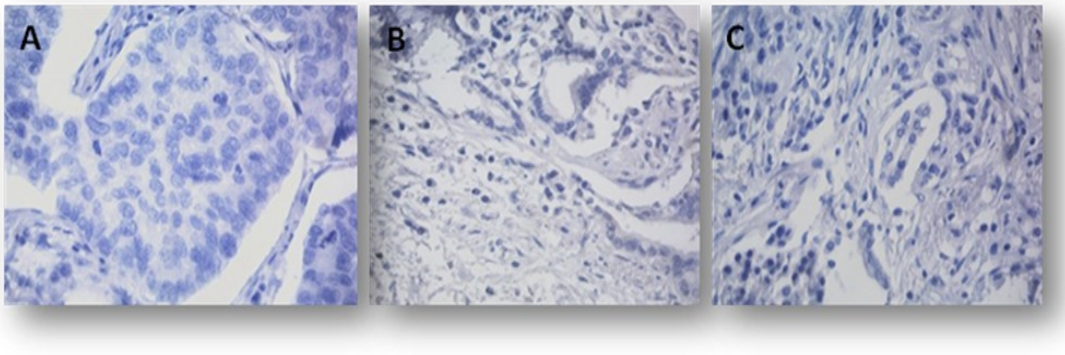
**D** (40X) the cytoplasm of all cancer cell has a very intense mark (score 3+)

**Image 8:** ADK, IHC ALK on tissue biopsies



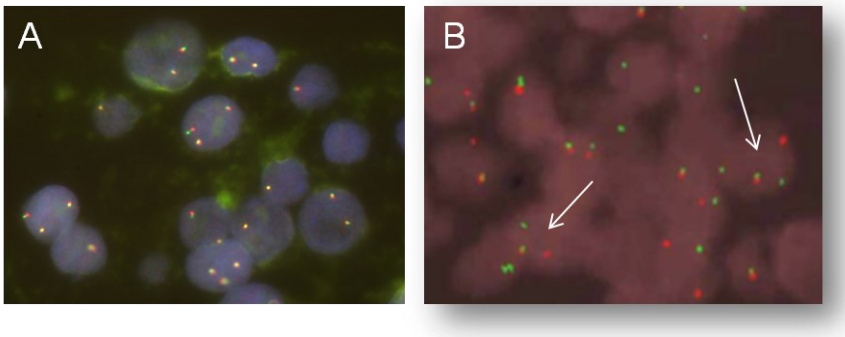

**A** (10X), **B** (20X) **C** (20X) the cytoplasm of almost all tumor cells has a weak mark (score 1+).

**Image 9:** ADK, IHC ALK tissue biopsies



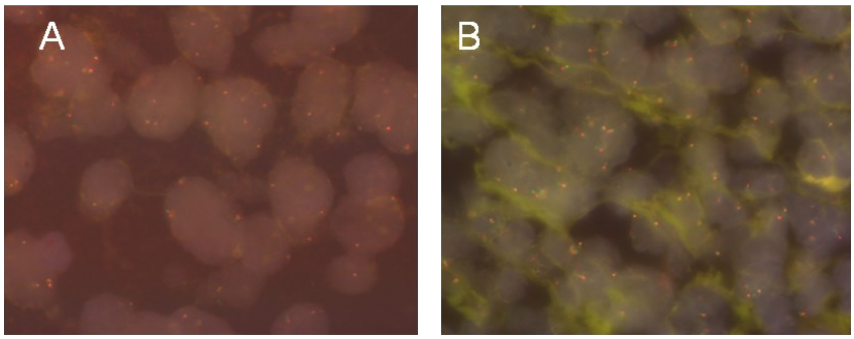

**A** (40X), **B** (20X) **C** (20X) the cytoplasm of tumor cells shows no marking (score 0).

**Image 10:** ADK, FISH *ALK Break-Apart probe on tissue biopsies*



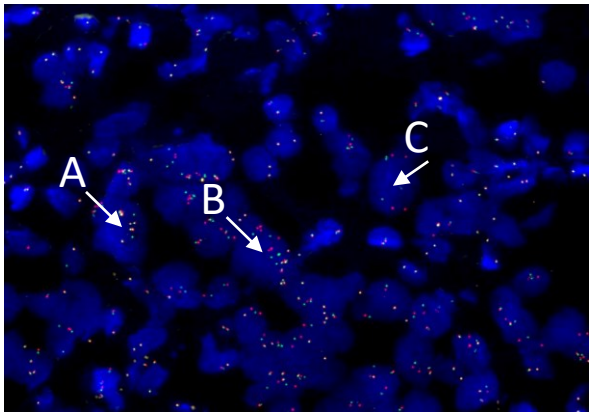

**A:** tumor cell nuclei (ADK) in the absence of ALK translocations (100X). **B:** nuclei of tumor cells (ADK) the absence of ALK translocations is noted, although some nuclei have a classic break-apart pattern (100X).

**Image 11:** ADK, FISH *ROS1 Break-Apart probe on tissue biopsies*




**A:** tumor cell nuclei (ADK) in the absence of *ROS1* translocations (100X). **B:** nuclei of tumor cells (ADK), the absence of *ROS1* translocations (100X) is noted.

**Image 12:** ADK, FISH *RET Break-Apart probe on tissue biopsies*




Tumor cell nuclei (ADK) with *RET* positive rearrangements for **A:** *RET* amplification with >5 fused signal/nuclei; **B:** both *RET* translocation and amplification with break-apart pattern and signals and >5 splitted signal/nuclei; **C:** *RET* classic break-apart pattern (40X).

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