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Association of circulating tumor DNA (ctDNA) mutations and circulating miRNAs variations, through liquid biopsy, in Non-Small Cell Lung Cancer (NSCLC) patients.

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Abstract

Lung cancer represents, to date, one of the leading causes of death worldwide in female and male population. To date, survival rate remains under 15% at 5 years of diagnosis. Indeed, the diagnosis of this tumor is unfortunately late on average 7 months after the onset of first symptoms, and most patients at the time of diagnosis have a locally advanced or metastatic disease. However, the approach of clinicians against pulmonary neoplasms has radically changed in the last years for the introduction of new targetable therapeutic strategies in clinical practice. In particular, 15% of Caucasian patients show an EGFR actionable mutation that makes them sensitive to tyrosine kinase inhibitors (TKIs). Although, to date, gold standard for EGFR molecular characterization is tissue, sometimes tissue biopsy does not seem to be the proper choice for the molecular characterization of the tumor. Therefore, in the need of new non-invasive approaches, liquid biopsy is proposed as a valid alternative in many cancers. In particular, in lung cancer liquid biopsy has completely entered in the clinical practice for the management and monitoring of cancer patients. Furthermore, recent AIOM guidelines contemplate the possibilty of replacing tissue biopsy in case of tissue unavalaibilty at diagnosis or at progression disease. In particular clinicians can now take advantage from the use of the third generation TKI Osimertinib, which is active also in T790M posistive patients that are resistant to first/second generation TKIs. This study represents a step forward for validating and recognizing the usefulness of liquid biopsy in clinical practice. Indeed, the study highlights the potentality of ctDNA in assisting oncologists in the proper therapeutic choise. As we presented in results the analysis of ctDNA allowed not only to unreveal T790M onset in no-responder patients but also allowed the identification of EGFR driver mutations at diagnosis in case of tissue unavailability. Moreover, we here provide evidence that cfDNA concentration may be an interesting surrogate biomarkers for the stratification of patients according to several features such as progression disease, diagnosis, gender and molecular loads in TKI and immunotheraphy treated patients. Furthermore, we studied the possible role of circulating miRNAs in a small cohort of NSCLC patients with a defined EGFR molecular profile. Although technical and standardization limitations, through a first *in vitro* approach then translated in patients, we found

a signature of miRNAs whose trend behaviour is correlable to EGFR molecular status and thus to clinical outcome. In particular, based on our experience, miR-30c, miR-126, miR-142-3p and miR-221, among the analyzed miRNAs, showed an interesting trend along the molecular history of NSCLC, with particular interest to their levels at T790M point. In conclusion, liquid biopsy analysis can be used in different moments of the disease starting from diagnosis to relapse, helping in obtaining a better patients' stratification with both prognostic and predictive value, and it can be a valuable and simple test to follow tumor response and moreover to identify resistance mechanisms. Furthermore, the concept of liquid biopsy is more actual than ever; indeed it meets the unstoppable demand of new needful biomarkers.

CHAPTER 1

Background, Rationale and Objectives

1.1 Lung cancer: Epidemiology and histopathological classification

Lung cancer is the leading cause of death worldwide with an alarming ever-increasing trend [1]. In Italy, the incidence and mortality rate for lung cancer has shown a markedly different trend by gender: in men population, there is a strong reduction in both incidence (-2% / year in recent years) and mortality (from 95 to 55 per 100,000 people per year) in relation to a remarkable reduction in smoking habits. This trend contrasts with that recorded in the female population, with a percentage of new cases of 2.7%/year between 1999 and 2016 (Linee guida Neoplasie del Polmone, AIOM 2017). However, it remains the first cause of death in men of all ages, while it is the second cause of death for tumors in women aged between 0-49 and 50-69 years (respectively 9% and 14%), representing the third most common cancer after the colon-rectum and breast. The average age of pulmonary cancer onset is between 40 and 70 years of age, with a peak around 50-60 years (only 2% of cases are diagnosed before 40 years). Survival rates, although increasing in recent years, remain very low (≤ 15% at 5 years of diagnosis) (SEER cancer Statistics Factsheets. Bethesda, MD: National Cancer Institute) due to the inability of diagnostic methods to highlight the disease at an early stage. In fact, the diagnosis of this tumor is unfortunately late, on average 7 months after the onset of symptoms, and 75% of patients at the time of diagnosis have a locally advanced or metastatic disease. Therefore, in order to improve the results obtained so far, it is essential to seek new therapeutic strategies, especially in patients with inoperable disease.

According to the criteria proposed by the World Health Organization (WHO), pulmonary neoplasms are mainly divided in two histotypes:

- Small Cell Lung Cancer (SCLC) or microcytoma accounts for about 10-15% of all pulmonary carcinomas, with a particularly unfavorable prognosis.
- Non-Small Cell Lung Cancer (NSCLC) (85%): Squamous or spinocellular carcinoma (25%), Adenocarcinoma (35%), non-differentiated large cell carcinoma (10%).

This classification is of great value for staging the pathology, for making the most appropriate therapeutic choice and prognosis formulation [2].

1.2 Biomolecular characterization

The carcinogenic process takes a few years, during which genetic mutations occur on genes (TP53, KRAS, BRAF, MYC) encoding proteins that control important cell functions such as differentiation, survival, apoptosis, and cell growth regulation. Specifically, *KRAS* and *BRAF* oncogenes, both involved in the EGFR mediated pathway, if altered, play a pivotal role in carcinogenicity of NSCLCs [3]. In particular, *KRAS* results predominantly mutated at codon 12 and codify for a GTPase involved in different signaling pathways such as PI3K-Akt-mTOR (cell survival) and MAPK-ERK (cell proliferation). In about 20-30% of pulmonary adenocarcinomas is the most frequent driver mutation, particularly in the Caucasian population [3], and has a negative prognostic value. The *BRAF* proto-oncogene, on the other hand, codes for a serine/threonine kinase involved downstream the signaling pathway mediated by *KRAS*, which regulates cell growth and differentiation. Furthermore, in today's clinical practice, the study of *ALK* (Anaplastic Lymphoma Kinase) and *EGFR* (Epidermal Growth Factor Receptor) alterations have become increasingly fundamental not only for their important prognostic value but also for their predictive role, representing, indeed, targetable therapeutic targets [4]. *ALK* mutations, rearrangements or amplifications can lead to the onset of different types of cancer, including NSCLCs. Specifically, the rearrangement of *ALK* with the Echinoderm Microtubule-Associated Protein Like 4 gene (EML-4), generates a chimeric kinase that leads to a constitutive activation of different cell growth pathways [3 5]. This translocation, identified for the first time in 2007, occurs in a subset (about 3-8%) of non-smoking patients diagnosed with adenocarcinoma [6]. Determination of EML4-ALK rearrangement is performed by FISH or immunohistochemistry, and is required to select patients to be treated with tyrosine kinase inhibitors (TKI) such as crizotinib [5]. *EGFR*, and other members of his family (HER-1), genetic alterations play an important role in the pathogenesis and progression of many types of cancer, including lung cancer. In fact, in most adenocarcinoma diagnoses, more than 80% of the

mutations identified occur into this gene [7]. EGFR is localized in the short arm of chromosome 7, consisting of 28 coding exons codifying for a 170 KDa transmembrane glycoprotein with tyrosine kinase activity (Figure 1). The binding of EGF ligand to its external portion of the receptor determine the dimerization which in turn activates a variety of pathways (MAPK, PI3K-AKT, JAK-STAT) implicated in survival, proliferation and cell growth mechanisms. As long as the ligand-receptor interaction is present, signal transduction persists until the receptor is inactivated [3]. In 40% of Asian patients and in about 10-15% of Caucasians with NSCLC diagnosis, EGFR is altered at the ATP binding pocket level, encoded by exons 18 to 21 (Figure 1 and 2). Therefore, alterations in these exons are defined as activators, since they cause de-regulation of the EGFR signaling, which remains in the active state. The most common activating mutations [3 8] are:

- Exon 18 point mutations (3%): G719C, G719S, G719A, and S720F;
- Exon 19 in frame deletions (Del19): about 20 different variants are known. The most frequent (48%) leads to the elimination of triplet-coding amino acids in positions 746-750;
- Exon 20 in frame insertions (Ex20INS), less common (4%), and S768I, T790M, C797S point mutations;
- Exon 21 point mutations: including p.L861Q and p.L858R. P.L858R is the second most frequent activating mutation (43% of EGFR mutated), consisting of a substitution of a leucine with arginine in position 858;

Among these, Del19 and p.L858R account for the 85-90% of cases of NSCLC [9]. Both are associated with increased sensitivity to first and second generation TKIs (gefitinib, erlotinib and afatinib) and their presence correlates with an objective response in 75% of the treated patients [10].

1.3 TARGET THERAPY: ALK and EGFR inhibitors

Unlike EGFR mutations, the presence of EML4-ALK rearrangement is an unfavorable prognostic factor. In 2011, crizotinib was approved by the Food and Drug Administration (FDA) as a treatment for ALK-positive NSCLC patients at progression [5]. It acts as a competitive ATP inhibitor that exerts the same mechanism of action of EGFR TKIs, however targeting the EML4-ALK fusion receptor (present in neoplastic cells). Various evidences have shown that mutations in the tyrosine kinase domain of the chimeric receptor lead to drug resistance. Therefore, as with the mutated EGFR counterpart, after about one year of treatment

with crizotinib, patients undergo therapeutic failure [11]. For this reason, new generation ALK inhibitors (alectinib and ceritinib) have been developed to inhibit tumor growth in this subset of patients [12].

The acquisition of new knowledge on EGFR structure and gene alterations has allowed the development of two different molecular target therapies: the first uses small molecules that can inhibit the EGFR mediated signaling pathway (gefitinib, erlotinib and afatinib); the other uses monoclonal antibodies (eg cetuximab, panitumumab) that inhibit ligand-dependent receptor activation [3]. Erlotinib and gefitinib were the first target molecules used as first-line treatment in non-smokers with locally advanced or metastatic disease. They are small molecules able to compete with ATP for binding to the catalytic site of mutated EGF receptors. This, thus, prevents receptor autophosphorylation, resulting in inhibition of downstream signaling and, consequently, proliferation of the mutated clone. Most patients show an initial response to first-generation TKI treatment, but despite this, approximately 50% of them inevitably undergo to drug resistance mechanisms. At this regard, new generation TKIs afatinib and osimertinib, second and third generation respectively, whose efficacy and tolerability have been investigated in several clinical trials, have been recently developed [13 14]. Afatinib has the same mechanism of action of first generation TKIs, but unlike them, it irreversibly binds to the ATP binding pocket. In fact, it appears to be a better second-line treatment choice in those patients with progression due to resistance mechanisms. Indeed, it has been widely demonstrated a statistically significant and clinically relevant benefit in terms of overall progression-free survival if compared to erlotinib [13]. Osimertinib, on the other hand, is an irreversible third generation TKI able to specifically target cell clones with resistance point mutations such as the T790M. In particular, Phase III AURA 3 study showed significant survival benefits in patients treated with osimertinib if compared with chemotherapy and first-generation TKIs treated patients at first line [14]. Because of the genomic instability of the tumor and its ability to adapt to micro-environment changes, cancer clones are able to undergo to resistance mechanisms. In fact, about 60% of patients after 7-12 months of TKIs administration (erlotinib, gefitinib or osimertinib) become non-responders, ie they no longer respond to treatment [13]. Therefore, several evidences have shown that duplications, insertions and missense mutations in several genes are associated with primary and / or acquired resistance to the aforementioned drugs [15]. Among the molecular alterations associated with secondary resistance are worthy of note:

- Amplification of c-MET (Mesenchymal-Epithelial Transition Factor). It is found in about 20% of NSCLCs;
- Amplification of HER2 (13%);

- Transformation in SCLC (3%);
- Point mutation of exon 20: p.T790M (50-60%), p.C797S

Among these, the most relevant and frequent clinical mutation, is the T790M, which consists in the substitution of a threonine with a methionine at aminoacidic position 790. The presence of this threonine at the ATP binding site is crucial for the specificity of the inhibitor. Indeed, its substitution with a more bulky methionine residue prevents TKI binding, leading, thus, to an increased ATP binding affinity. However, recent clinical trials have shown that in a small subset of patients with adenocarcinoma (about 3% of cases), this mutation is already present at the time of diagnosis (primary resistance) in combination with one of the activating mutations mentioned above [13]. Instead, in about half of no longer responders to TKIs administration, this mutation was found. This data supports the hypothesis that the onset of the T790M in some cell clones could be stimulated by the selective pressure operated by TKIs.

1.4 Immunotherapy in NSCLC

Immunotherapy can be easily defined as the use of targeted medicine to stimulate a person's own immune system to recognize and kill cancer cells more effectively. In fact, in order to survive and escape the immune system, tumor cells can adopte several strategies at various steps of the dynamic immunoediting process such as the down-regulation or the enhancement of inhibitory molecules on immune and tumor cell surface, low antigen presentation on tumor cells, increased production of interleukin-10 and transforming growth factor-β (TGF-β) with consequent lower maturation of dendritic cells [16]. In the latest years, the clinical development of immune checkpoint inhibitors represents the main step forward in the treatment of advanced non-oncogene addicted non-small cell lung cancers (NSCLCs) [17]. In particular, some clinical features, such as smoking history, the absence of driver mutations and the presence of specific cancer subtypes, seem to be associated with better response to immunotherapy [18] [19]. The best known immune checkpoint receptors are cytotoxic T-lymphocyte antigen-4 (CTLA-4) expressed on T cells in early activation stage, and programmed cell death protein-1 (PD-1) mainly expressed on T cells in effector phase but also on B cells and natural killer (NK) cells. (Figure 3). In particular, Nivolumab represents a second-line treatment option for squamous non-small cell lung cancer. It acts by blocking a negative regulator of T-cell activation and response, thus allowing the immune system to attack the tumor. In fact, when PD-L1 or PD-L2 binds to PD-1 receptor, T cell becomes inactived, avoiding thus the immune system to fight tumor cells. Nivolumab blocks PD-L1 from binding to PD-1, allowing the T cell to work.

1.5 LIQUID BIOPSY IN NSCLC

Currently, tissue biopsy remains the gold standard for the diagnosis and follow up of patients with NSCLC [20]. However, this procedure has several disadvantages because, in addition to being highly invasive, the Formalin-fixed Paraffin-embedded (FFPE) tissue can lead to DNA degradation and the samples is often poorly satisfactory or aceptable in terms of quantity and quality for molecular analysis. Furthermore, tissue biopsy is unable to capture the heterogeneity of the tumor, as each tumor clone, under the selective pressure of the micro-environment, can accumulate a large number of mutations in genes that play a crucial role in maintaining the balance between growth and cell death [21]. In fact, a biopsy provides only a snapshot of the tumor at site and time of sampling, and may not contain all the tumor subclones, thus representing a sampling bias. All of these reasons justify the strong interest of the scientific and oncological community towards less invasive techniques such as liquid biopsy [21]. Moreover, the molecular analysis of the tumor and/or of the metastatic lesions is becoming more and more requested not only at diagnosis but also at progression disease (PD). Therefore, a liquid biopsy can be easily repeated at different time-points allowing to follow the tumor molecular status during the treatment course [15]. This could help clinicians to predict disease progression over time, to identify new acquired molecular alterations and to observe how all these characteristics corresponde to patient's status. (Figure 4) There are many definitions of "liquid biopsy". The definition is complex since different body fluids as urine, ascites, saliva, cerebrospinal liquid or plasma can be considered as valuable sources of tumor components. Currently, for patients with pulmonary adenocarcinoma, the use of liquid biopsy for mutational investigations is a rapidly expanding field, both in the field of translaction research and in the field of molecular oncology diagnostics. This method, allow to directly detect tumor-specific biomarkers as well as exosomes, circulating tumor cells (CTCs) and circulating tumor nucleic acids, including microRNA, RNA and circulating tumor DNA (ctDNA).

1.5.1 Circulating tumor DNA (ctDNA)

Circulating tumor DNA (ctDNA) represents the fraction of cfDNA deriving from the tumor mass. Several studies have suggested that ctDNA is derived from the combination of apoptotic processes that release DNA fragments of between 180-200 bp and necrotics which instead give rise to longer DNA fragments due to random digestion of the genomic DNA [22 23]. To date, its

release mechanisms are still partially unknown and need to be deeply investigated. Moreover different tumor types do not release the same amount of ctDNA and, even in patients with the same disease, the concentration of ctDNA may vary consistently [24]. The easiest way to identify the ctDNA is to investigate the presence of somatic driver mutations, which, by definition, can be exclusively found on tumor. In fact, circulating DNA extracted from the plasma of a patient with neoplasia always contains tumor-specific mutations [25]. Recently, the AIOM Guidelines have approved the use of ctDNA for the determination of mutagenic state of EGFR if tissue / cytology testing can not be performed due to the lack of suitable neoplastic material. Some studies have shown that ctDNA may be a viable alternative to monitor the response to TKIs in mutated EGFR tumors and to detect early resistance mechanisms, such as T790M mutation, several months before clinical evidence of progression of disease [14 26]. In addition, molecular analysis of ctDNA during patient follow up is a much less invasive technique than classic tissue biopsy and allows early detection of disease recovery and tumor metastasis, so that rapid intervention in the choice the most appropriate therapeutic. Although tumor tissue remains the one for molecular characterization of tumors, the data obtained support the clinical utility of liquid biopsy both for predictive purposes and for monitoring response to pharmacological treatment. The biggest limitation of this technique is that the ctDNA, compared to the entire DNA content present in the plasma released by healthy cells (free circulating DNA), represents a small fraction of DNA of tumor origin. Indeed, in biological fluids (urine, plasma, serum) the free tumor free DNA concentration is normally low and may vary within a range of 1-100 ng / ml. So the biggest challenge in research is to increase the amount of ctDNA in the bloodstream. This limitation could lead to false negatives due to the low amount of circulating alleles. For this purpose, new sensitive technical approaches are available to analyze EGFR mutational status from plasma-derived ctDNA. In particular, digital PCR (dPCR) and Next Generation Sequencing (NGS) platforms represent to date the most studied approaches for application in clinical practice. The information arising from ctDNA analysis will broad from early diagnosis to prognosis as well as response to drug administration and real time monitoring of the disease.

1.5.2 Diagnostic role of ctDNA

To date, several studies and meta-analysis deeply highlighted the diagnostic value of plasma-based EGFR testing in NSCLC patients, showing an interesting accuracy of ctDNA in terms of sensitivity and specificity if compared with the gold-standard tissue genotyping [27 28] . Therefore, the isolation of ctDNA from plasma or serum would be helpful for EGFR testing in

all those patients whose tissue is not available at diagnosis or tissue analysis results are inconclusive. Sacher et al. have recently evaluated the reliability of plasma analysis. This study demonstrated a high specificity (100%) and sensitivity (74-82%) in 80 patients with advanced NSCLC harboring activating EGFR del19/L858R mutations using droplet digital PCR (ddPCR) [29]. The same promising results at diagnosis have been also showed within the multicenter ASSESS study in which a similar concordance rate of 89% (sensitivity 46%, specificity 97%) has been found in a cohort of 1162 patients with advanced NSCLC [30]. Furthermore, despite Real-Time PCR and ddPCR techniques are definitely, the most used for ctDNA analysis, NGS is emerging as an important tool that can complement or substitute tissue NGS analysis. Indeed, there are several commercially available NGS panels specifically designed for ctDNA testing in lung cancer. Recently Villaflor et al. assessed the utility of two ctDNA panels in a clinical series of 68 NSCLC patients; the 54-gene panel include only mutations whereas the 68-gene include also *ALK, RET* or *ROS1* fusions [31]. In this paper, it was also investigated the concordance between paired tissue and blood samples whenever possible. The results reported that 80% of patients have detectable ctDNA, with 83% presenting at least one non-synonymous ctDNA alteration. As expected the most frequent mutations were reported in TP53, KRAS and EGFR genes [31]. These results suggest that, ctDNA analysis may also be applied in early stage disease.

1.5.3 Prognostic role of ctDNA

The prognostic role of ctDNA has been deeply investigated. In 2014 the group of Wang et al. tested the ability of dPCR to identify T790M in plasma ctDNA compared to a non-digital approach (ARMS). They showed a statistical correlation between survival and allele fraction of circulating T790M before and after EGFR-TKI administration. Patients with increasing levels of circulating T790M during EGFR TKI treatment showed better progression free survival (PFS) and overall survival (OS) if compared with patients who do not displayed any significant T790M variation [32]. Furthermore, in 2016 the same research group confirmed that patients with circulating T790M had a better clinical outcome compared to plasma T790M-negative patients [33]. Recently, Thompson et al. correlated survival with ctDNA levels and number of variants using NGS in plasma specimens of metastatic NSCLC patients. The high levels of ctDNA (>3ng/mL), irrespective of mutational profile, were associated with decreased survival. Conversely, patients with ctDNA levels lower than 3ng/mL showed a better median survival (24 months vs. 46 months, respectively). Furthermore, OS seems to be strictly correlated with

number of variants detected in plasma. Indeed, a number of variants greater than 3 determined an OS reduction from 62 months to 46 months, giving thus a poorer prognosis [34]. Therefore, it seems that mutational load itself may be a good prognostic marker.

1.5.4 ctDNA value in real time monitoring of the disease

The translation in clinical practice of liquid biopsies is strictly requested in all those cases in which a disease progression monitoring is needed. Indeed, on November 2015 the FDA approved osimertinib as new treatment option for patients with metastatic EGFR T790M-positive NSCLC patients who failed prior EGFR-TKI [35] [36]. Patients' selection is strictly based on the identification of T790M mutation and for the first time the molecular analysis can be performed either through tissue re-biopsy or in plasma sample [37] [38]. The non-invasive potential of ctDNA has been deeply studied by Oxnard in many studies specifically focused on the molecular biology of NSCLC. In 2014, one of the first study performed by its group highlighted the possibility to anticipate clinical evidence of progression through early molecular evidences. Indeed, the analysis of ctDNA through ddPCR, in serial plasma sampling, allowed the detection of resistance mutations (T790M) weeks and sometimes months prior to radiological progression [39].

Recently his group prospectively evaluated the sensitivity and specificity of plasma genotyping by ddPCR in 180 patients with advanced NSCLC, including 60 patients with acquired resistance to EGFR-TKI. Plasma genotyping by ddPCR exhibited 79% specificity and 77% sensitivity in the detection of T790M mutation, which are lower than those observed with EGFR-activating mutations at baseline. In addition Oxnard et al. showed that outcomes of T790M-positive patients included in the phase I AURA study were similar if T790M was detected in plasma or tumor tissue. Conversely both RR and PFS of T790M-negative patients on plasma were significantly higher than T790M-negative on tissue, and further tumor genotyping of plasma T790M-negative patients allowed to identify a subgroup of T790M-positive patients on tumor tissues who had better outcomes. According to these data the authors suggest that plasma genotyping could represent the first step for the detection of T790M status at the time of PD. However, because of the low sensitivity (70%) of the current available technologies, which are associated with a 30% false negative rate, patients with T790M-negative on plasma should repeat tumor tissue biopsy to further investigate the presence of such molecular alteration [40].

1.6 Circulating Tumor Cells (CTCs)

The circulating tumor cells are shed from both primary and metastatic tumor, thus they are representative of the tumor from which they detached. It is known that lung cancer releases a limited number of CTCs and therefore they were not so far considered a good field of study. Nevertheless, limitations of CTCs detection in lung cancer were mainly due to the limited available isolation methods. Thanks to the increase of knowledge about CTCs biological and physical characteristics, detection and isolation methods have been consequently improved. Nowadays, CTCs may become a promising field of study also in lung cancer [41].

CTCs can be used for two different aims: to evaluate the risk of metastasis and as a source of nucleic acid for molecular characterization. Indeed, CTCs are shed to the blood stream and can play an important role in the metastatic process. Moreover, since CTCs spread directly from the tumor they might harbor the same mutational landscape that can be investigated through molecular analysis. The studies on CTCs in lung cancer have shown heterogeneous results, mainly due to the different techniques and criteria used for the experiments. Tanaka et al. demonstrated that the number of CTCs is higher in patients with lung cancer than in those with benign disease, and the number of CTCs is significantly increased in patients with distant metastasis than in the primary ones. In the same study the authors demonstrated a significant correlation between the number of CTCs in the blood stream and the stage of the disease [22], but other studies have not showed the same results [42] [23]. The number of CTCs can be also a good marker of tumor growth and prognosis. Krebs et al. demonstrated that patients with 5 or more CTCs in 7.5 mL of total blood, after one cycle of chemotherapy, have a worse prognosis as compared to those with a lower number [43].

1.7 Circulating microRNAs

MicroRNAs are evolutionarily conserved, endogenous small non-coding RNAs with 18-25 nucleotides that have important functions in diverse biological processes, such as cell proliferation, differentiation, and apoptosis [44]. The promising role of circulating miRNAs as disease biomarkers has been deeply evaluated and still continues to increase the interest of scientists. However, technical aspects of miRNAs isolation, measurement and quantification still represent the critical steps of circulating miRNAs analysis. Indeed, sample processing, isolation, hemolysis in blood samples, the lack of stable reference gene as well as the wide variety of genome platforms are only a few of the many not negligible aspects [45]. In circulating

miRNAs analysis the first and pivotal step is to identify a feasible source of nucleic acids. As reported in Weber et al study the most common source of circulating miRNAs are plasma, serum, urine, and saliva but also microvescicles and exosomes [46]. Even if the exosomal miRNAs can probably provide more information, their isolation is complex [47]. Isolation of circulating miRNAs from plasma or serum is easier despite the high content of blood components in these body fluids. Furthermore, plasma and serum specimens often show a different spectrum of miRNAs also within the same individuals. Serum seems to be better source for miRNA isolation because the yield of miRNA is greater than the one obtained from plasma; this is probably due to the contamination of RNAs deriving from platelets during the clotting process [48]. Also in plasma the levels of miRNA could be influenced by hemolysis as recently reported by Kirschner et al. In fact, miR-16 and miR-451 plasma levels are highly increased as usually they are in blood cells [49] [50]. Generally, the concentration of miRNAs in body fluids is very low. Therefore, the isolation and enrichment of miRNAs is an extremely delicate and important procedure. Nowadays, for the RNAs isolation we can rely on manual extraction methods as the phenol/chloroform or commercially distributed kits. Overall, they show differential efficiency even if the phenol/chloroform method showed higher yields (400ng / 500μL of plasma) if compared to the commercial kits (50ng / 200μL of plasma) [51] [52].

1.8 Treatment algorithms in tissue or plasma-based wild type or EGFR mutated NSCLCs

Nowadays, at diagnosis, to decide the proper therapeutic approach in advanced non-squamous NSCLC, international guidelines recommend to test tumor DNA for EGFR mutation. The current gold-standard remains tissue biopsy, even if it's limited by several features, such as the not easy access to different tumor sites, the invasiveness of procedures,the tumor heterogeneity, and not ultimately the low patients' compliance (Figure 5). Thus, in the last decade an alternative not invasive approach, known as liquid biopsy, has been proposed to overcome the aforementioned issues. An increasing number of studies and meta-analysis evaluated the diagnostic value of plasma-based EGFR testing in the management of NSCLC patients, overall showing a sensitivity of 0.62 and a specificity of 0.96 as compared with the standard tissue genotyping, which suggest an adequate diagnostic accuracy of circulating tumor (ct)DNA analysis [27] [28] [53] [54]. These evidences have led to the analytical validation and the clinical approval of EGFR mutation testing by using ctDNA isolated from plasma or serum of about 30% of patients whose tissue is not available at diagnosis or tissue analysis results are not evaluable (Figure 6). Recently a growing interest has been focused also on monitoring dynamic

changes in plasma of both sensitizing and resistant EGFR-mutations during TKI treatment. Particularly after the clinical approval of osimertinib in United States, Europe and Japan, re-biopsy at progression became mandatory, in order to identify T790M mutation or other alternative mechanisms of acquired resistance and ultimately personalize second-line therapy. The non-invasive potential of ctDNA has been deeply studied by Oxnard in many studies. In 2014, he first demonstrated that the analysis of ctDNA through ddPCR in serial plasma sampling during TKI treatment, allowed the detection of the resistance T790M mutation weeks and sometimes months prior to radiological PD, highlighting the possibility to anticípate clinical evidence of progression through early molecular evidences [1] [39]. Figure 6 describe the algorithm used in NSCLC patients at progression.

1.8 Objectives

The aim of this project is to verify the feasibility of liquid biopsy in NSCLC clinical practice and it is an interesting attempt to study new circulating biomarkers (miRNAs). In particular, the study has been divided in two parallel approaches. In the first approach we evaluated, in two cohorts of NSCLC patients, with a different EGFR molecular status, the effectiveness of cfDNA as prognostic and predictive tool. The second approach is a study conducted firstly in vitro, on EGFR wt/mutated NSCLC cell lines, through the identification of a signature of de-regulated miRNAs involved in the disease, then traslated on patients. In particular, the miRNA signature, its circulating levels and trend along the EGFR molecular status, has been analyzed in the plasma of healthy patients, EGFR wild type, EGFR Del19 and EGFR T790M NSCLC patients.

CHAPTER 2

Materials/Patients and Methods

2.1 Patients' selection and plasma collection

Patients enrolled in the present study were recruited at the Medical Oncology Department of the University General Hospital "Paolo Giaccone" in Palermo. The study was conducted on a double cohort of patients all diagnosed with NSCLC. In the first cohort we included 60 metastatic patients (histotype adenocarinoma), elegible or not for TKIs administration; this cohort was further divided in two groups: progression disease (PD) subgroup (34 patients), diagnosis (D) subgroup (26 patients) (table 2 and 3). In the second cohort were enrolled 41 patients, all diagnosed with NSCLC, both squamous and non-squamous histotypes, under immunotherapy administration with Nivolumab. We have further collected samples from 3 healthy subjects that were used for circulating miRNA analysis. Characteristics of patients selected for this part of the study are listed in table 1. Patients' samples collection was performed according to national legislation concerning ethical requirements. Written informed consents were obtained from all study participants and the samples were analyzed anonymously. All control patients were enrolled after exclusion of any pathological conditions. Blood samples were collected in 3 vacutainer tubes containing EDTA and processed within 2 hours from withdrawal, in order to avoid the release of DNA from nucleated blood cells. The plasma was carefully separated from the cell fraction through two successive centrifugations: the first, performed at low speed (1200 g x 10 minutes at 4°C) to avoid cell lysis, leads to the separation of the plasma from the corpuscular part of the blood; the second one at 3000 g x 10 minutes at 4°C, is generally performed to eliminate residual cellular debris and red blood cells from the plasma. After these two centrifugations, the plasma was transferred into new tubes and, after being properly coded, it was stored at -80°C until further molecular analysis.

2.1.2 Cell-free DNA extraction and quantification

Starting from a volume of 1 mL of plasma, circulating DNA was exctracted through the commercial kit QIAamp® Circulating Nucleic Acid, Qiagen) (Figure 7), which uses a vacuum pump to increase extraction speed and efficiency. The extraction was performed following the manufacturers' instructions. Cell-free DNA was eluted in 55 µl of Buffer AVE and stored at -20°C. DNA quantification was performed using the Qubit™ dsDNA HS Assay kit (Invitrogen, Life Technologies, CA, USA) in a Qubit 3.0 fluorometer (Invitrogen, Life Technologies, CA, USA) (Figure 8). This instrument calculates the concentration of DNA present in a sample on the basis of the fluorescence emitted by a fluorophore, which binds specifically to double-stranded DNA (dsDNA), by comparing the concentration to that of the standards provided in the kit. For this reason it is a specific and sensitive test, as it allows to detect DNA concentrations between 0.01-100 ng/µl.

2.1.3 Chip-based digital PCR (dPCR)

To analyze the EGFR exon 19-20-21 mutational status (Del19, L858R, T790M) from ctDNA within the first cohort (PD/D), was used the QuantStudio™ 3D Digital PCR (dPCR) platform (Thermofisher). The dPCR was performed in a final volume of 16 µL as follow: 4 µL of cfDNA (cfDNA concentration 2 ng), 8 µL of QuantStudioTM 3D dPCR Master Mix (Thermofisher), 0,4 µL of wild type conjugating VIC-probe, 0,4 µL of target conjugating FAM-probe, 1,44 µL of 10 µM forward primer, 1,44 µL of 10 µM reverse primer and 0,32 µL of nuclease-free water. Sample mix was loaded on a chip using the QuantStudio 3D Digital PCR Chip loader (Thermofisher) and then PCR reaction was performed on ProFlex™ 2× Flat PCR System (Thermofisher) with the following cycling conditions: 96 °C for 10 min, 45 Cycles (2 min at 60°C and 30 sec at 98°C) and a final hold at 60°C for 2 min. After PCR amplification steps, QuantStudio™ 3D Digital PCR chips were first read through QuantStudio™ 3D Instrument and then analyzed through the QuantStudio™ 3D Analysis Suite Software (Thermofisher) which provide an absolute quantification.

The QuantStudioTM 3D Digital PCR was also used to confirm the miRNAs expression pattern obtained with Real Time-PCR in *in vitro* experiments. The analyses were conducted in triplicate using circulating miRNAs isolated from NSCLC patients listed in table 1. The dPCR was performed in a final volume of 15 µL as follow: 2.25 µL of cDNA (cDNA concentration 2 ng), 8 µL of QuantStudio™ 3D dPCR Master Mix (Thermofisher), 4 µL of nuclease-free water and 0.75 µL of TaqMan MicroRNA Assay (Thermofisher). Sample mix was loaded on a chip using the QuantStudio 3D Digital PCR Chip loader (Thermofisher) and then PCR reaction was

performed on ProFlex™ 2× Flat PCR System (Thermofisher) with the following cycling conditions: 96°C for 10 min, 40 Cycles (2 min at 56°C and 30 sec at 98°C) and a final hold at 60°C for 2 min. After PCR amplification steps, QuantStudio™ 3D Digital PCR chips were first read through QuantStudio™ 3D Instrument and then analyzed through the QuantStudio™ 3D Analysis Suite Software (Thermofisher) which provide an absolute quantification of miRNAs present in the samples.

2.1.4 Statistical analysis

The Students' t-test was used for the study of cfDNA in the PD/D cohorts. The Mann Whitney test was used for intergroup comparisons of two indipendent samples in the immunotherapy cohort. A p-value <0.05 was used as a threshold for statistical significance. Survival analysis was performed using Kaplan-Meier method, providing median and p-value.

2.2 *IN VITRO* EXPERIMENTS: Lung adenocarcinoma cell cultures

A549 (EGFR wild type), HCC827 (exon 19 E746-A750 deletion) and resistant H820 (exon 19 E746-A750 deletion + exon 20 p.T790M, c.2369C>T) cell lines were purchased from ATCC (Manassas, VA) and were cultured in RPMI1640 (GIBCO) with 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin/streptomycin (GIBCO) in a controlled atmosphere (5% CO_2) at 37°C. Furthermore, HCC827 cell lines were subjected to long-term culture for 6 months, in presence of TKI Erlotinib (Selleckchem) properly re-suspended in DMSO (SIGMA). Viability assay was carried out by plating 4000 cells/well into black-bottom 96-well plates. The following day, cells were drugged with TKI across a 4 dose range, 10nM, 100nM, 1 μM and 10μM. 24 and 72 hours post drug treatment, cell viability was measured using MTT assay (Promega) (Figure 9) HCC827, thus named tHCC827, on the basis of IC50, were grown in increasing doses of Erlotinib for 6 months starting from 10nM and increasing to 50 and 100nM incrementally once the cells began to grow through the given dose.

2.2.1 *IN VITRO* EXPERIMENTS: miRNAs profiling

miRNAs were extracted from cultured cells using miRNeasy® Mini Kit (QIAGEN) and quantified using a NanoDrop spectrophotometer. The reverse transcription (RT) was performed using a TaqMan® microRNA Reverse Transcription Kit (Applied Biosystems) and Megaplex RT primers (Human Pool A, Applied Biosystems) following the manufacturer's instructions. RT was performed on a 9700 thermocycler (Applied Biosystem) with the following cycling conditions: 40 cycles at 16°C for 2 min, 42°C for 1 min and 50°C for 1 s followed by a final step of 85°C for 5 min to inactivate reverse transcriptase. Megaplex™ RT products were mixed with RNase-free water and TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2X (Applied Biosystem) and then loaded into TaqMan Human MicroRNA Array A (Applied Biosystems). Real-time PCR-based microfluidic card with embedded TaqMan primers and probes in each well for the 380 different mature human miRNAs and controls has been performed on ABI PRISM 7900HT system (Applied Biosystems) using the 384-well TLDA default thermal-cycling conditions: 50°C for 2 min, 94.5°C for 10 min, 97°C 30 sec followed by 40 cycles at 95°C for 30 s and 59.7°C for 1 min. The cycle threshold was automatically calculated using SDS 2.4 software (Applied Biosystems) and miR-191 was used as an endogenous control. The relative expression levels of miRNAs were calculated using the comparative ΔΔCt method and fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta Ct}$.

2.2.2 *IN VITRO* EXPERIMENTS: Single miRNAs assay

Reverse transcription was performed for miR191, miR30c, miR100, miR126, miR140, miR142-3p, miR221 and miR495 using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). A total of 15 µl reactions were incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real time RT-PCR was performed on ABI Prism 7900HT System (Applied Biosystems) at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The qRT-PCR reactions were all performed in triplicate. The cycle threshold was automatically calculated using SDS 2.4 software (Applied Biosystems) and miR-191 was used as an endogenous control. The relative expression levels of individual miRNAs were calculated using the comparative ΔΔCt method and the fold changes were calculated by the equation $2^{-\Delta\Delta Ct}$.

2.3.1 TRANSLATIONAL STUDY: Circulating total RNAs extraction

Starting from a volume of 1 mL of plasma total circulating RNAs were exctracted through the commercial kit QIAamp® Circulating Nucleic Acid (Qiagen) (Figure 7), which uses a vacuum pump to increase extraction speed and efficiency. The extraction was performed following the manufacturers' instructions. Total circulating RNAs were eluted in 30 µl of Buffer AVE and stored at -20°C.

CHAPTER 3

Results

3.1 Evaluation of actionable and resistance EGFR mutations in ctDNA from NSCLC patients

EGFR actionable and resistance mutations have been investigated on ctDNA of 60 NSCLC patients through dPCR. The cohort has been further divided in two different subgroup. In the first group only patients in progression disease (PD) have been enrolled (n. 34, Table 2) whereas in the second only patients at diagnosis (D) are included (n.26, Table 3). After TKIs administration many patients experience treatment failure; one of the main cause of TKI efficacy failure is represented by the onset of T790M resistance mutation. Interestingly the new national AIOM guidelines for lung cancer establish that T790M mutation can be investigated in liquid biopsy in a patient that experienced PD under TKI treatment. Moreover, in the same guidelines, it is clearly stated that in case of tissue unvaiability at diagnosis liquid biopsy can be a valid alternative to investigate EGFR actionable mutation. Consequently in case of mutation detection patients can benefit from TKI administration.

Here we resume the main relevant characteristics of patients belonging to the PD subgroup, the detailed information are reported in table 2:

- Concerning sex frequency we found a not-equal distribution among female/male cases. In fact, we found a percentage of 62 (21/34) for women versus 38 for men (13/34).
- Concerning EGFR mutation frequency we found: Del19 74% (25/34), L858R point mutation 23% (8/34), Ins20 3% (1/34).
- With the liquid biopsy analysis we reported that in the PD gruup 53% of the cases (18/34) showed a concordance between tissue and ctDNA mutational status.

- In addition, in 47% of cases (16/34) T790M resistance mutation was diagnosed, responsible for the ineffectiveness of TKIs treatment. Moreover in 69% (11/16) of T790M+ patients, the resistance mutation is accompanied by the activating mutation.

Here we resume the main characteristics of patients belonging to the D subgroup, the detailed information are listed in table 3:

- Concerning sex frequency we found a not-equal distribution among female/male cases. In fact, we found a percentage of 31 (8/26) for women versus 69 for men (18/26).

- EGFR activating mutations detected in ctDNA account for 15% of cases (4/26), in accordance to what previously reported. In detail we have identified two patients harboring Del19 and two patients with L858R point mutation. Furthermore, in one case at D we detected a very rare double mutation L858R+T790M.

By mean of the Students' t-test we have investigated the possible correlation between cfDNA concentration (ng/mL) and some cohorts' features and molecular characteristics. As aspected, median cfDNA concentration resulted, higher in the PD cohort (1.578,24 ng/mL) respect to the D (733,46 ng/mL) cohort. Furthermore, in both cohort cfDNA levels appeared to be consistently correlable with gender; indeed, despite the limited statistical significance, female population showed a greater cfDNA concentration compared to male population and this correlation seems to be more evident in the PD cohort. cfDNA concentration evaluation seems to be a valuable surrogate biomarkers also looking at the mutational load; in fact in correspondance of a greater mutational load (Del19/L858R+T790M) there is also a greater cfDNA concentration (2.760 ng/mL). This result is in line with the concept that a metastatic disease has a greater representativeness in bloodstream than a controlled disease in terms of mass burden and molecular spread (Figure 10).

3.1.2 Prognostic role of cfDNA in a cohort of EGFR wild type patients under immunotherapy treatment.

In the last years immunotherapy represents a new valid therapeutic option for EGFR wild type NSCLC patients. Unfortunately, to date no circulating biomarkers are available to monitor patients along their clinical history given the non-oncogene addicted characteristic of the disease. Therefore, in this study we aimed to analize cfDNA concentration as a surrogate biomarker.

Table 4 summarizes the characteristics of the 41 patients included in our study. In particular, they are mainly male, aged under 70, current or former smoker, with non-squamous histology. Moreover, 49% of patients reported a PD at first instrument evaluation, of them 47% showed an overall survival lower than 6 months from treatment initiation identifying a subgroup of poor prognosis patients. Interestingly in this subgroup we reported the highest level of cfDNA both at treatment initiation and along treatment.

The median cfDNA levels in the 41 patients evaluated are 0.47 ng/μL at baseline and 0.56 ng/μL after 4 cycles of therapy administration (Figure 11).

In the presented study, we have also correlated cfDNA levels with other clinical features. Indeed, we observed that more than 80% of patients with baseline cfDNA levels greater than 0.47 ng/μL, reported a progression disease at their first re-evaluation. Conversely, only 26% of patients with baseline cfDNA levels lower than 0.47 ng/μL experienced progression disease. Furthermore, 74% of patients with cfDNA levels lower than 0.47 ng/μL at baseline showed an appreciable disease control rate (DCR) compared to those with cfDNA levels greater than 0.47 ng/μL (17% of DCR). (Figure 12)

Similarly, at follow-up we observed that 93% of patients with cfDNA levels greater than 0.56 ng/μL, reported a progression disease at their first re-evaluation. Conversely, only 24% of patients with cfDNA levels lower than 0.56 ng/μL experienced progression disease. Furthermore, 76% of patients with cfDNA levels lower than 0.56 ng/μL during follow-up showed an appreciable DCR compared to those with cfDNA levels greater than 0.56 ng/μL (7% of DCR). (Figure 13)

We have also aimed to study progression free survival (PFS) and OS in patients stratified according to cfDNA levels at baseline and follow-up. Indeed the Kaplan-Meier curves demonstrate that both PFS and OS are worse in the subgroup of patients with higher cfDNA

levels at baseline and at follow-up (Figure 14). Conversely, patients with lower cfDNA levels showed a better outcome. Moreover, at follow-up, statistical significance is higher than the baseline counterpart, suggesting that during treatment cfDNA levels can better discriminate patients who benefit from treatment.

Finally, we stratified patients on the basis of cfDNA dynamic changes during treatment and we observed that patients with a > 20% increase had a significantly lower PFS and OS than those with no increase, suggesting a practical and useful cut-off for clinical practice after proper validation (Figure 15).

3.2 *IN VITRO* EXPERIMENTS: miRNAs array profiling

To obtain new insights on the molecular signature of metastatic NSCLC we investigated its miRNA expression profile. In particular, a screening analysis and relative quantification of 377 modulated miRNAs was performed through real time PCR in four NSCLC cell lines (A549, HCC827, tHCC827 and H820) simulating the most common EGFR mutational condition of this neoplastic disease: wild type, exon19 deletions and exon20 T790M single nucleotide variants. miRNAs were analysed using TaqMan MicroRNA Assays (Panel A Cards, Thermofisher Scientific) in four cell lines (A549, HCC827, H820 and tHCC827) and miR191 was used as endogenous control. Following Real-Time PCR experiments, analysis was performed through the SDS 2.4 software (Applied Biosystems) by crossing different experimental conditions. In details the three most interesting combinations were (Figure 16):

- A549 *vs.* H820: by using A549 as calibrator we reported, in H820, 43% up-regulated miRNAs and 40% down-regulated miRNA; 17% miRNAs were undetermined as Ct values were higher than 35
- HCC827 *vs.* H820: by using HCC827 as calibrator we reported, in H820, 47% up-regulated miRNAs and 30% down-regulated miRNA; 23% miRNAs were undetermined as Ct values were higher than 35.
- HCC827 *vs.* tHCC827: by using tHCC827 as calibrator we reported, in HCC827, 52% up-regulated miRNAs and 17% down-regulated miRNA; 32% miRNAs were undetermined as Ct values were higher than 35.

In particular, a fold change greater than two was used as cut-off to identify significantly deregulated miRNAs. Based on further and more detailed literature review, we decided to

proceed our investigation by focusing on those miRNAs reported to be significantly up/down regulated. In fact, we identified a signature of 8 miRNAs (miR30c, miR100, miR126, miR140, miR142-3p, miR143, miR221, miR495) possibly involved in NSCLC with different EGFR genotypes.

3.2.1 *IN VITRO* EXPERIMENTS: single miRNA validation

After the identification of the most de-regulated miRNAs as previously described, we further validated the miRNA expression pattern using single TaqMan assay for the specific miRNAs. The results obtained are showed in figure 17 and miR191 was used as endogenous control. MiR-126, -142-3p, -143 and -221 show a peculiar expression; indeed. as showed in figure 17, all these miRNAs are increased in T790M+ cell line (H820) except for miR-221 where the levels are similar to those reported in long-term treated cell line (tHCC827). MiR-30c, -100, -140 and -495 showed an opposite trend, indeed these miRNAs are consistently less expressed in T790M+ cell line.

3.3 TRANSLATIONAL STUDY: circulating miRNAs in NSCLC patients

On the basis of the results obtained *in vitro*, we decided to concentrate and analyze only those miRNAs whose trend seemed to be more interesting concerning disease outcome. We then analyzed circulating miR-30c, miR-126, miR-142-3p and miR-221, through dPCR, in 4 different groups: 3 healthy controls, 3 EGFR wild type NSCLC patients, 3 EGFR Del19 NSCLC patients and 3 EGFR T790M NSCLC patients. Patient's characteristics are reported in table 1. The bar graphs in figure 18 show the results achieved with these experiments. In this case miRNAs concentration is expressed in copies/μL as mean value for each group (healthy control, WT, Del19 and T790M).

The results showed in figure confirm an interesting trend of these four miRNAs along the different molecular pattern of NSCLC. Indeed miR-126, miR-142-3p and miR-221 increase is greater in T790M+ patients, showing an interesting growing trend. Conversely, miR-30c showed the opposite behaviour; indeed, the copies/μL decrease reaching the lowest value in T790M+ patients. Furthermore, the graph in Figure 19 is a merge of the aforementioned miRNAs, in terms of copies/μL, along the EGFR molecular status of the disease. MiRNAs

fluctuations seem to be characteristics in oncogene-addicted disease. As showed in figure 19, in healthy controls and WT patients the selected miRNAs do not significantly fluctuate. Contrariwise, in Del19 and T790M+ patients there is a peculiar and significant modification according to mutational status. As previously mentioned, miR-126, miR-142-3p and miR-221 show the same growing trend while miR-30c seem to slightly decrease until resistance.

CHAPTER 4

Discussion and conclusions

Lung cancer represents, to date, one of the leading causes of death worldwide with an alarming increasing trend. Despite the advent of new therapeutic and diagnostic approaches, survival rate remains under 15% at 5 years of diagnosis, due to the inability of screening methods to highlight the disease at an early stage. The diagnosis of this tumor is unfortunately late, on average 7 months after the onset of symptoms, and 75% of patients at the time of diagnosis have a locally advanced or metastatic disease. However, the approach of clinicians against pulmonary neoplasms has radically changed in the last years. Indeed, the discovery of targetable genes, in particular in NSCLC, has deeply modified the clinical course of the disease with the introduction of new therapeutic strategies. In the last decade the introduction of precision medicine in clinical practice allowed to reach new important goals in terms of OS and PFS. Furthermore, cancer patients can benefit from greater drug tolerability and thus enjoy a "healthier" lifestyle. Nowadays, the molecular characterization of a pletora of genes (EGFR, KRAS, BRAF, ALK) represents the starting mandatory point in the management and monitoring of NSCLC patients. Indeed, the 15% of Caucasian patients show an EGFR actionable mutation that makes them sensitive to tyrosine kinase inhibitors (TKIs). To date, the gold standard for EGFR molecular characterization is the tissue deriving from a biopsy or a surgical resection. Unfortunately, sometimes "the tissue is the issue" because of the poor availability of the sample, the not easy access to the tumor site and not ultimately the low patients' compliance. Moreover, at progression, tissue biopsy does not seem to be the proper choice for the molecular characterization of the tumor. Therefore, in the last decade a new non-invasive approach has been introduced in the clinical practice for the management and monitoring of cancer patients. In particular, the use of liquid biopsy in lung cancer clinical routine has deeply revolutionized the attitude of clinicians towards the disease. Furthermore, recent AIOM guidelines contemplate the possibilty of replacing tissue biopsy in case of tissue

unavalaibilty at diagnosis or at progression disease. In particular clinicians can now take advantage from the use of the third generation TKI Osimertinib, which is active also in T790M posistive patients that are resistant to first/second generation TKIs (erlotinib, gefitinib and afatinib).

Nowadays, big efforts are still needed to improve the molecular characterization of a highly heterogeneous tumor and to find new biomarkers. In particular, it's necessary to introduce new highly sensitive diagnostic methods able to discriminate poorly represented mutated alleles in a wide range of wild type fragments. To this purpose, digital PCR and NGS could represent the new ambrosia for overcoming the aforementioned limits. The presented study represents a step forward for validating and recognizing the usefulness of liquid biopsy in clinical practice. Indeed, the study highlights the potentality of ctDNA in assisting oncologists in the proper therapeutic choise. As we presented in results the analysis of ctDNA allowed not only to unreveal T790M onset in 47% of no-responder patients but also allowed the identification of EGFR driver mutations at diagnosis in 15% of cases where no tissue was available.

As previously mentioned, liquid biopsy and specifically ctDNA analysis is a decision-making tool as clinician can modify treatment approach according to ctDNA result specifically in T790M patients where the third generation TKIs is available. Another important aspect is the possibility to offer a better therapeutic chance for those patients that, at diagnosis, do not have enough tissue to perform EGFR molecular testing. Moreover, with our work we provide evidence that the simple cfDNA concentration may be an interesting surrogate biomarkers for the stratification of patients according to several features such as progression disease, diagnosis, gender and molecular loads. Immunotherapy represents the main breakthrough in NSCLC treatment after the introduction of TKIs. Nevertheless there is still the lack of prognostic, predictive and surrogate biomarkers for the management of patients treated with immunotheraphy. In our work we therefore investigated the possible value of cfDNA dynamic modifications during Nivolumab treatment to predict patients outcome. cfDNA concentration seems to reliably help in stratifying patients for several clinical features such as overall survival, progression free survival and disease control rate. Furthermore, we here suggest a practical and useful cut-off for clinical practice.

As often mentioned, liquid biopsy represents an incredible source of putative new interesting biomarkers. Among these, circulating miRNAs could represent in a nearby future a new possible reality in oncology. Indeed, tha fact that miRNAs exert a regulatory role towards the expression of many genes involved in cancer, make

themselves undisputed players of the tumor event. Unfortunately, many efforts are still needed to improve their applicability for many technical and standardization reasons. Although actual limitations, this project unrevealed the great potentiality of circulating miRNAs. In fact, through a first *in vitro* approach then translated in patients, we found a signature of miRNAs whose trend behaviour is correlable to EGFR molecular status and thus to clinical outcome. In particular, based on our experience, miR-30c, miR-126, miR-142-3p and miR-221, among the analyzed miRNAs, showed an interesting trend along the molecular history of NSCLC, with particular interest to their levels at T790M point. Moreover, miR-30c trend is in accordance with a previous data, not yet published, of a study conducted on exosome-derived miRNAs through qPCR. In fact, in this study circulating miR-30c levels, from circulating exosomes, have been assessed on a NSCLC patient underwent to TKI resistance. In particular, higher levels of miR-30c have been found during treatment and lower levels were detected after progression disease at T790M onset. However, further validating studies are still needed and mandatory.

In conclusion, the incorporation of cfDNA analysis can definitely improve lung cancer patients' management because it can provide a better molecular stratification even when tissue cannot be obtained due to ethical and safety reasons. Liquid biopsy analysis can be used in different moments starting from diagnosis to relapse, earning multiple clinical meanings. In fact, at diagnosis, it can help in obtaining a better patients' stratification with both prognostic and predictive value, rather than during treatment, and it can be a valuable and simple test to follow tumor response and moreover to identify resistance mechanisms. Therefore it is clear that liquid biopsy has already improved NSCLC patients' management as it offers a noninvasive but valid method to detect actionable and resistance mutations. Furthermore, the concept of liquid biopsy is more actual than ever; indeed it meets the unstoppable demand of new needful biomarkers.

CHAPTER 5

Tables and Figures

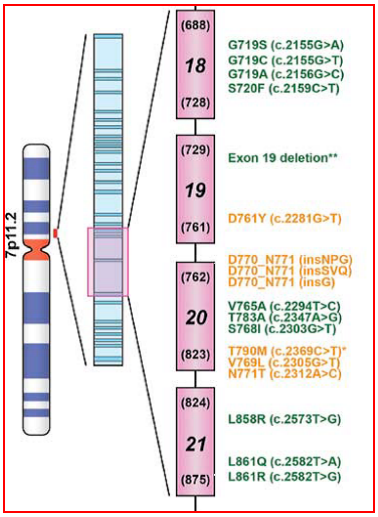

Figure 1: chromosome position of EGFR gene and actionable mutations found in EGFR exons 18, 19, 20 and 21

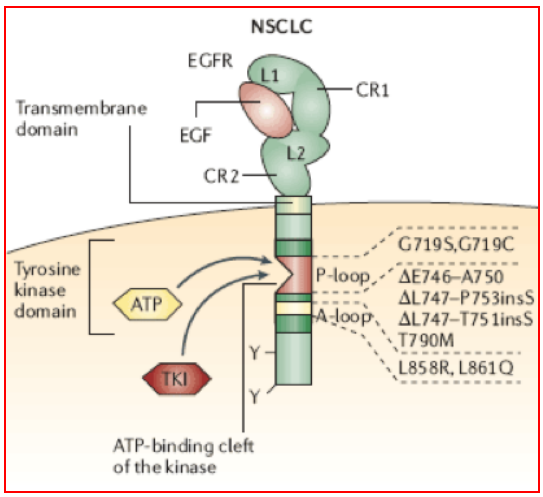

Figure 2: schematic representation of the EGFR structure and possible mutations in the ATP binding pocket

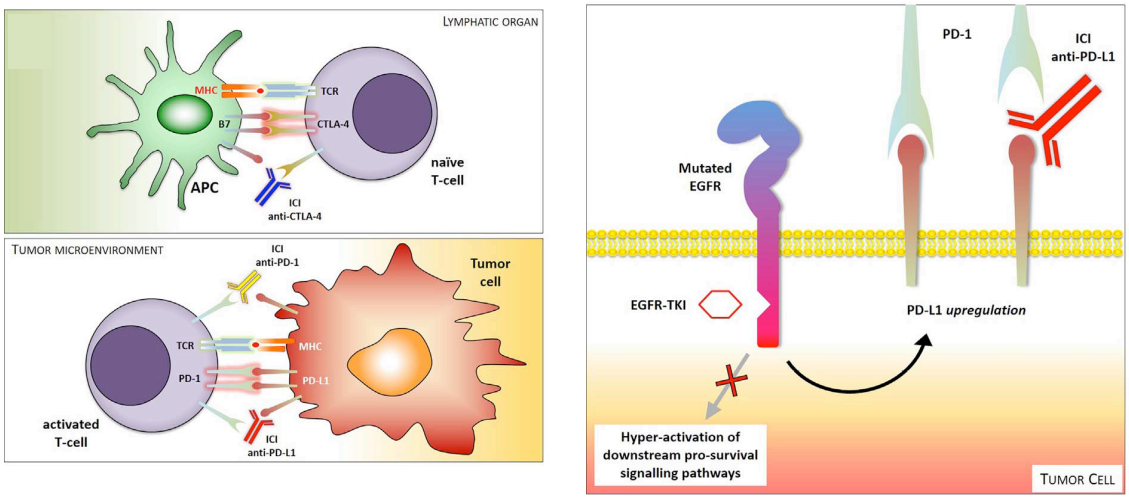

Figure 3: the action mechanisms of immune checkpoint inhibitors

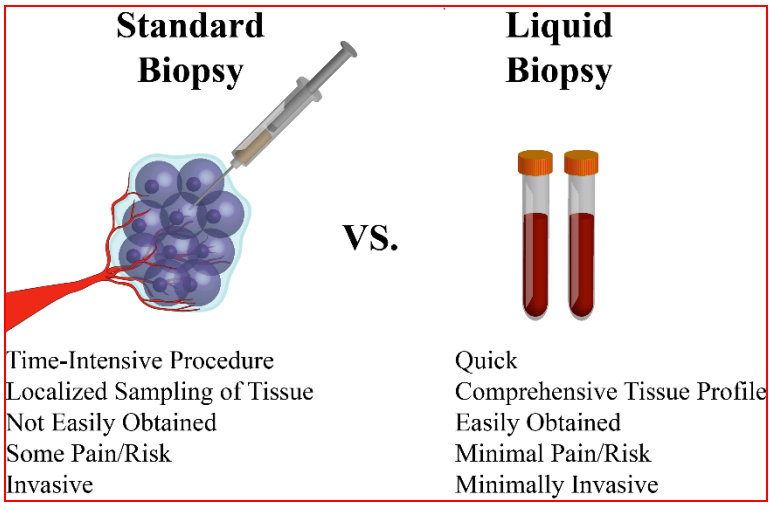

Figure 4: standard biopsy versus liquid biopsy

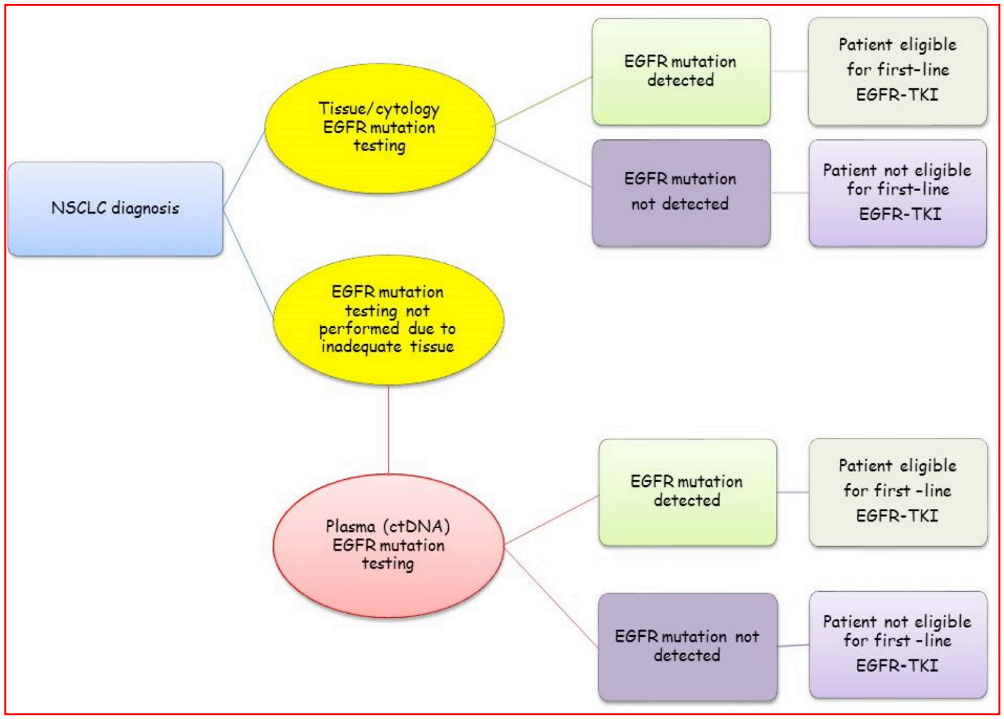

Figure 5: The role of plasma (ctDNA) EGFR mutation testing at the time of NSCLC diagnosis

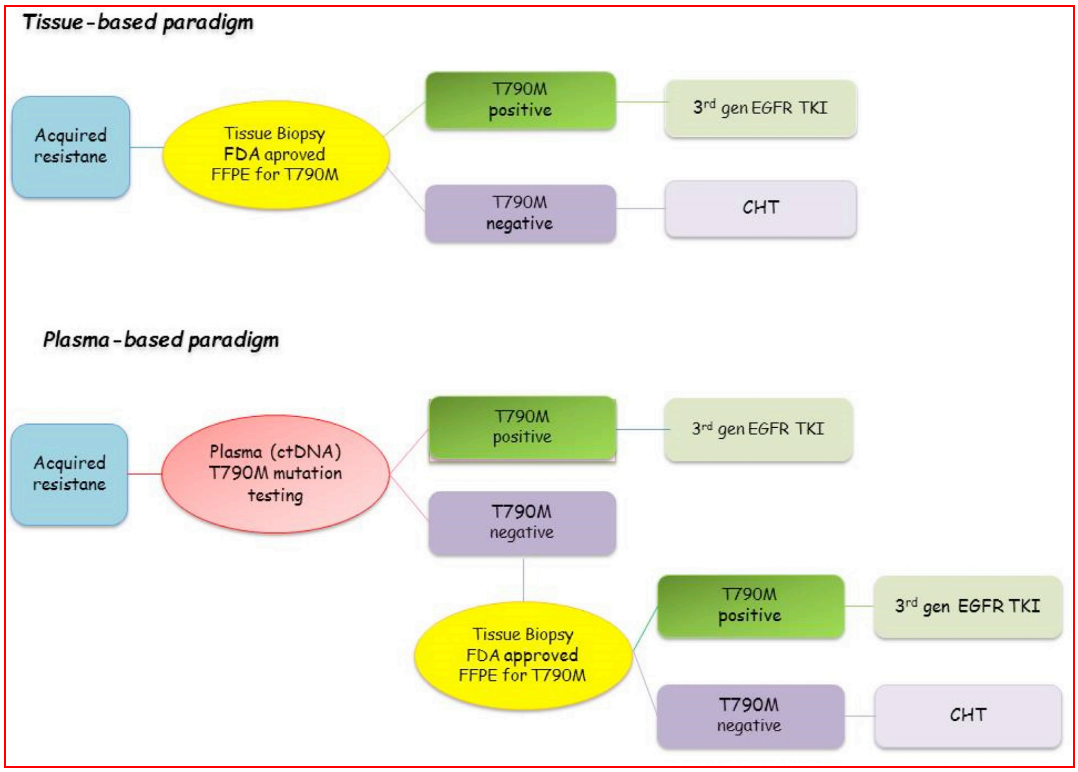

Figure 6: the role of plasma (ctDNA) EGFR-T790M mutation testing at the time of NSCLC progression

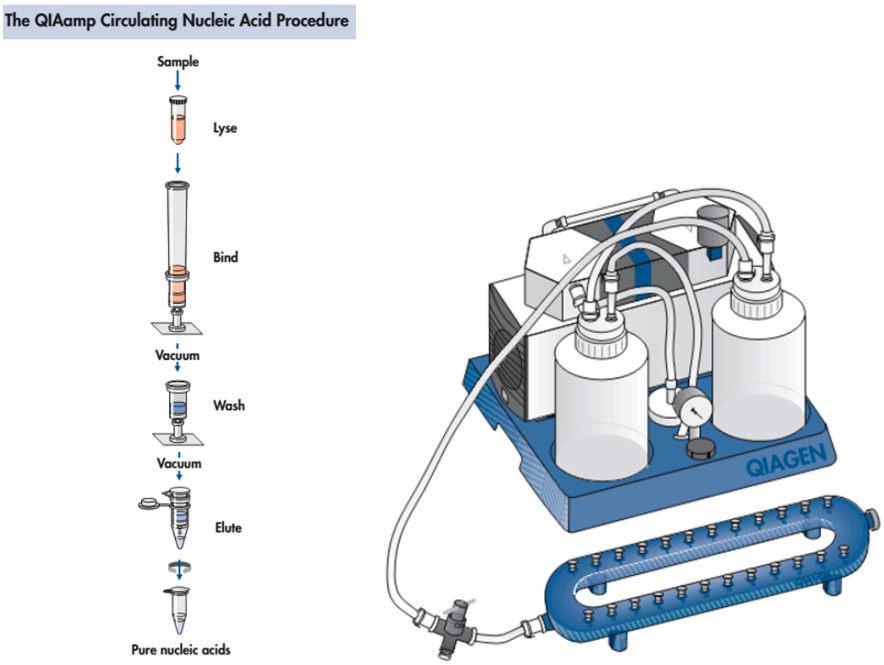

Figure 7: QIAamp® Circulating Nucleic Acid, Qiagen – procedure and vaccum pump system

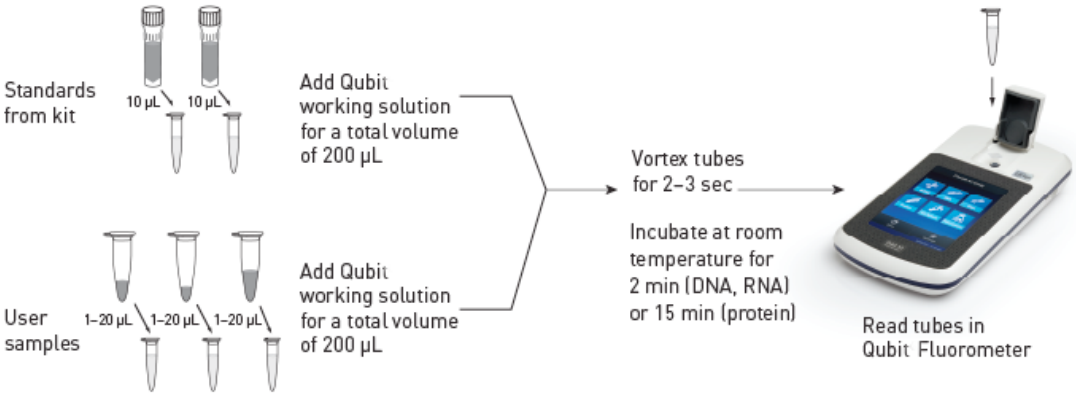

Figure 8: Qubit™ dsDNA HS Assay workflow and Qubit 3.0 fluorometer

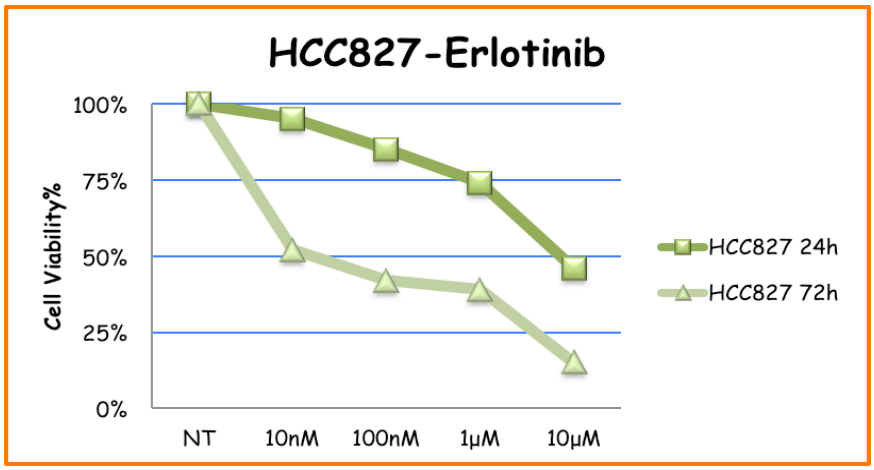

Figure 9: MTT assay performed on HCC827 cell line to establish cell viability to incremental Erlotinib concentrations.

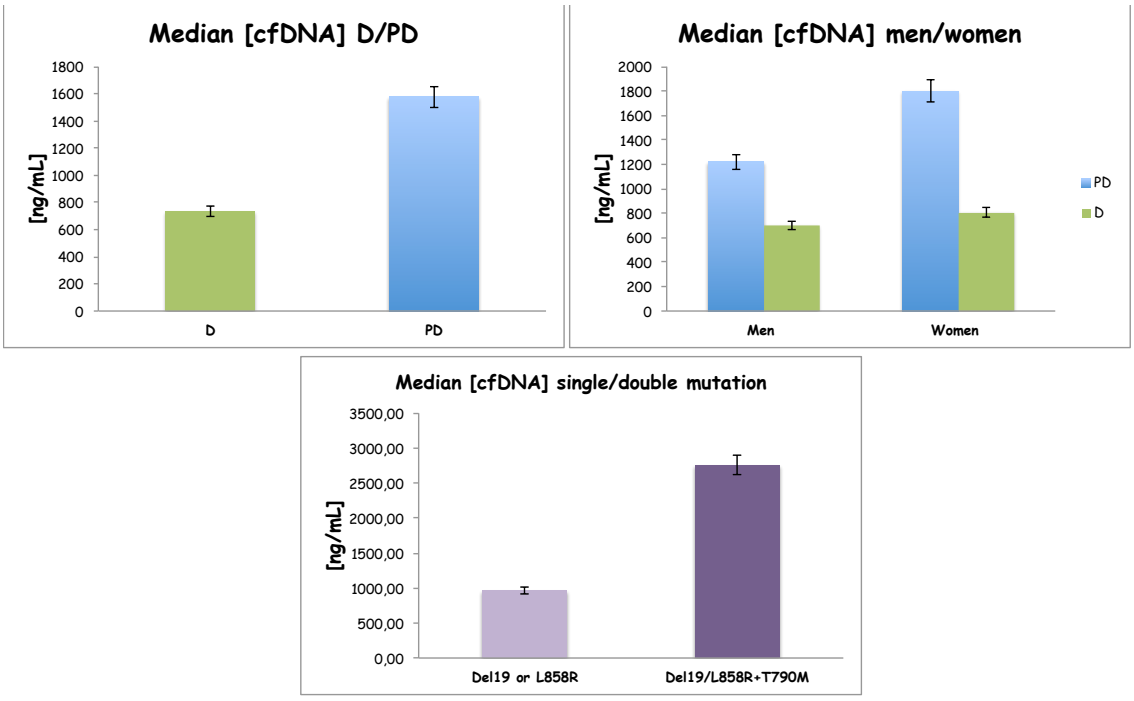

Figure 10: cfDNA concentration value in patients' stratification for PD/D, gender and mutational load.

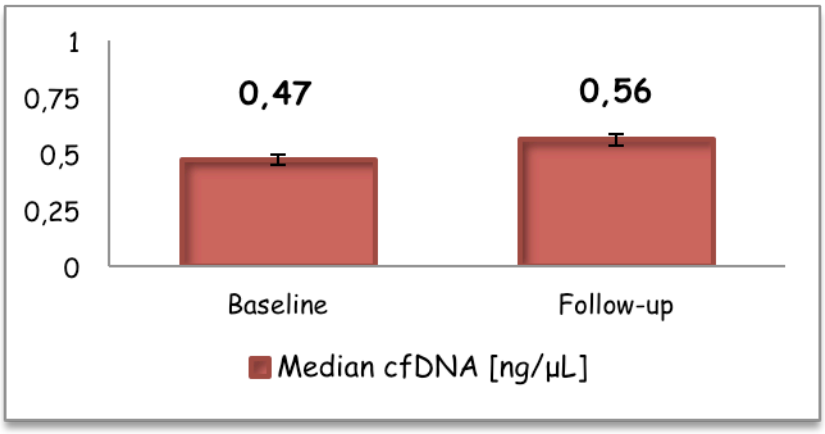

Figure 11: median cfDNA concentration [ng/µL] at baseline and follow-up in Nivolumab treated patients.

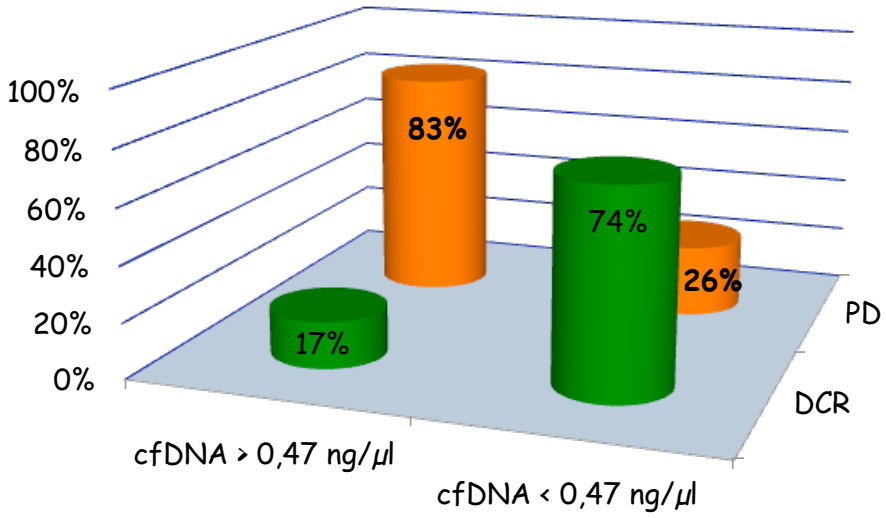

Figure 12: disease control rate (DSR) and progression disease (PD) according to the median cfDNA at baseline.

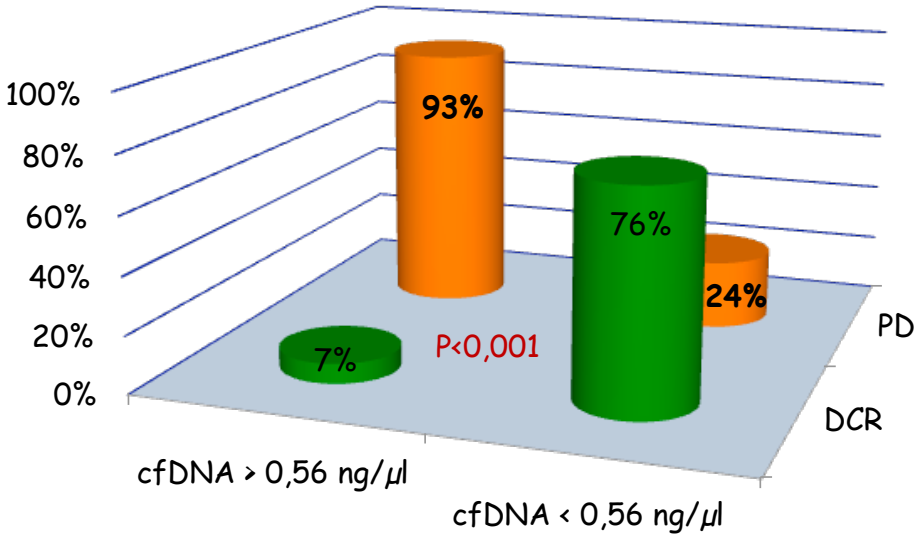

Figure 13: disease control rate (DSR) and progression disease (PD) according to the median cfDNA at follow-up.

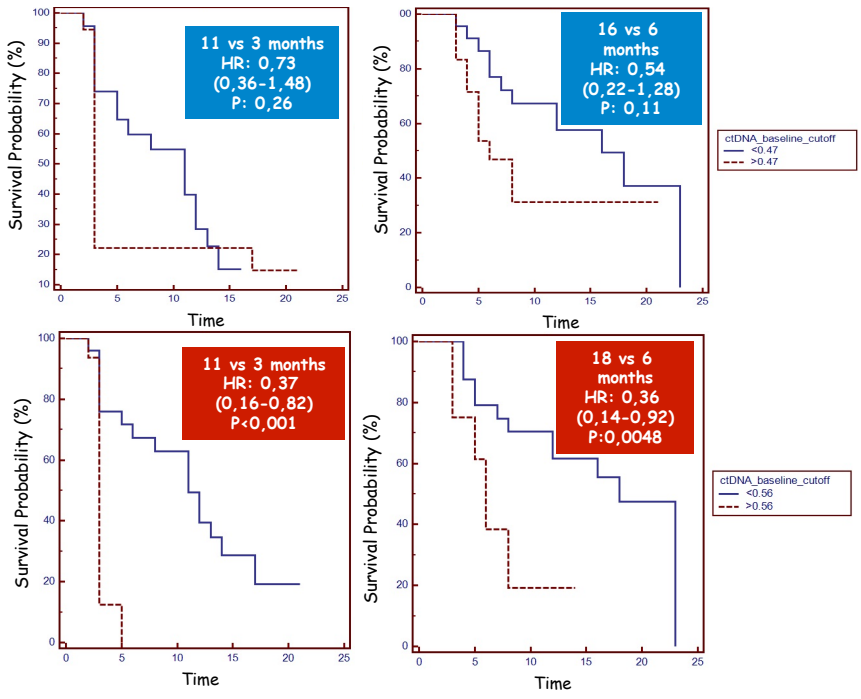

Figure 14: PFS and OS according to the median cfDNA at baseline (upper) and follow-up (lower). In the upper panel red line indicates a cfDNA concentration > 0,47ng/µl, the blue line indicate a concentration < 0,47ng/µl. In the lower panel the red line indicates a cfDNA concentration > 0,56ng/µl, the blue line indicate a concentration < 0,56ng/µl.

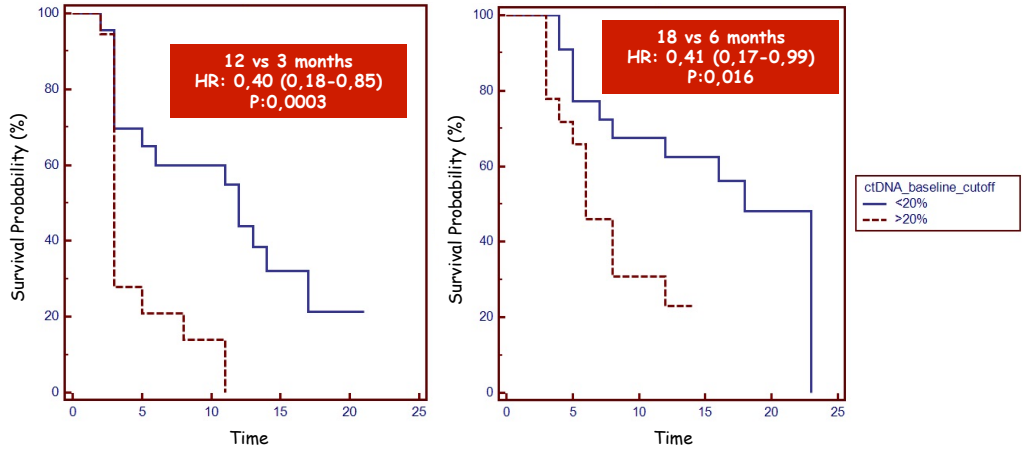

Figure 15: PFS and OS according to the median cfDNA increase during follow-up. The red line indicates > 20% increase in cfDNA.

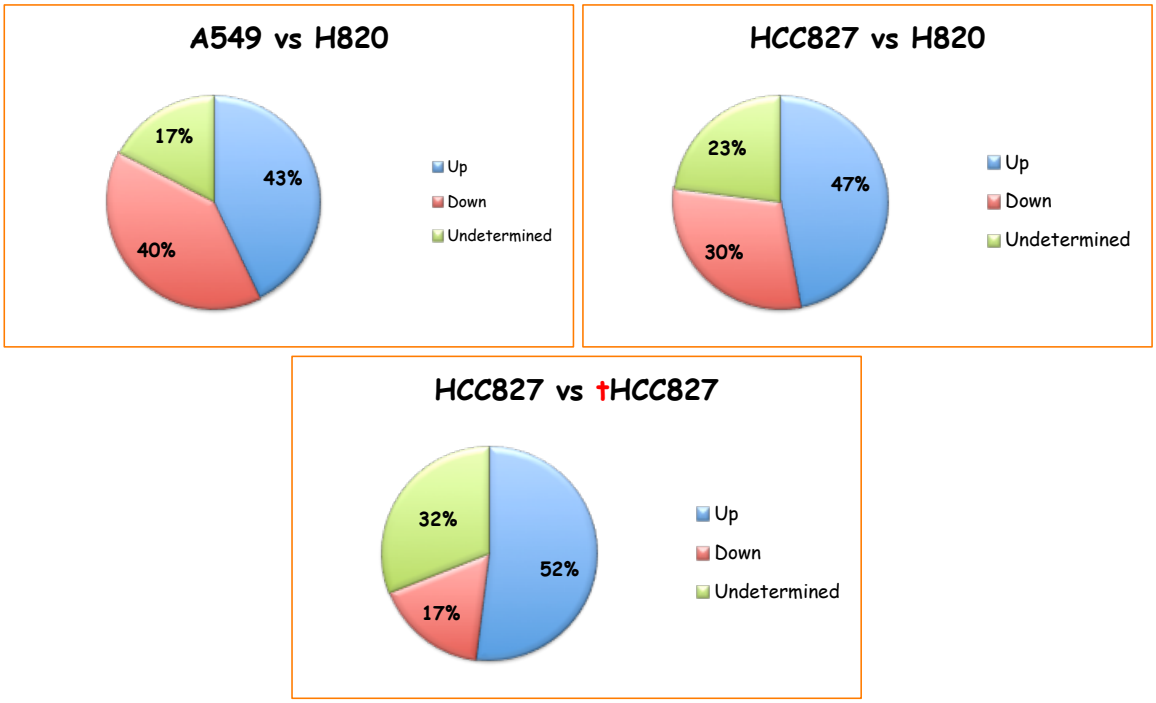

Figure 16: pie chart showing the percentage of up/down/undetermined miRNAs in the A549-H820, HCC827-H820, HCC827-tHCC827 card combinations.

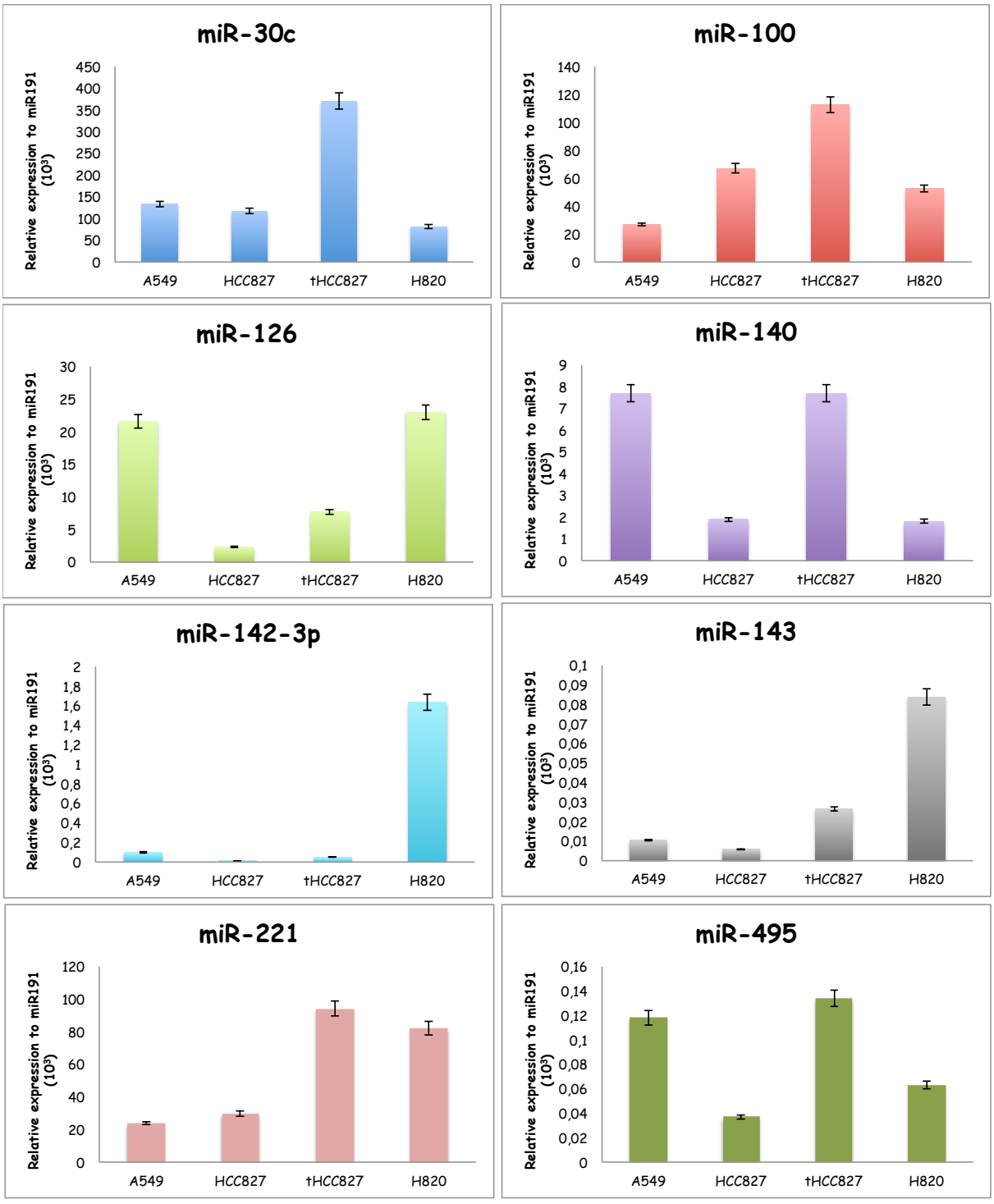

Figure 17: bar graph showing the expression of miR30c, miR100, miR126, miR140, miR142-3p, miR143, miR221, miR495 re-tested in single assay in A549, HCC827, tHCC827 and H820 cell lines.

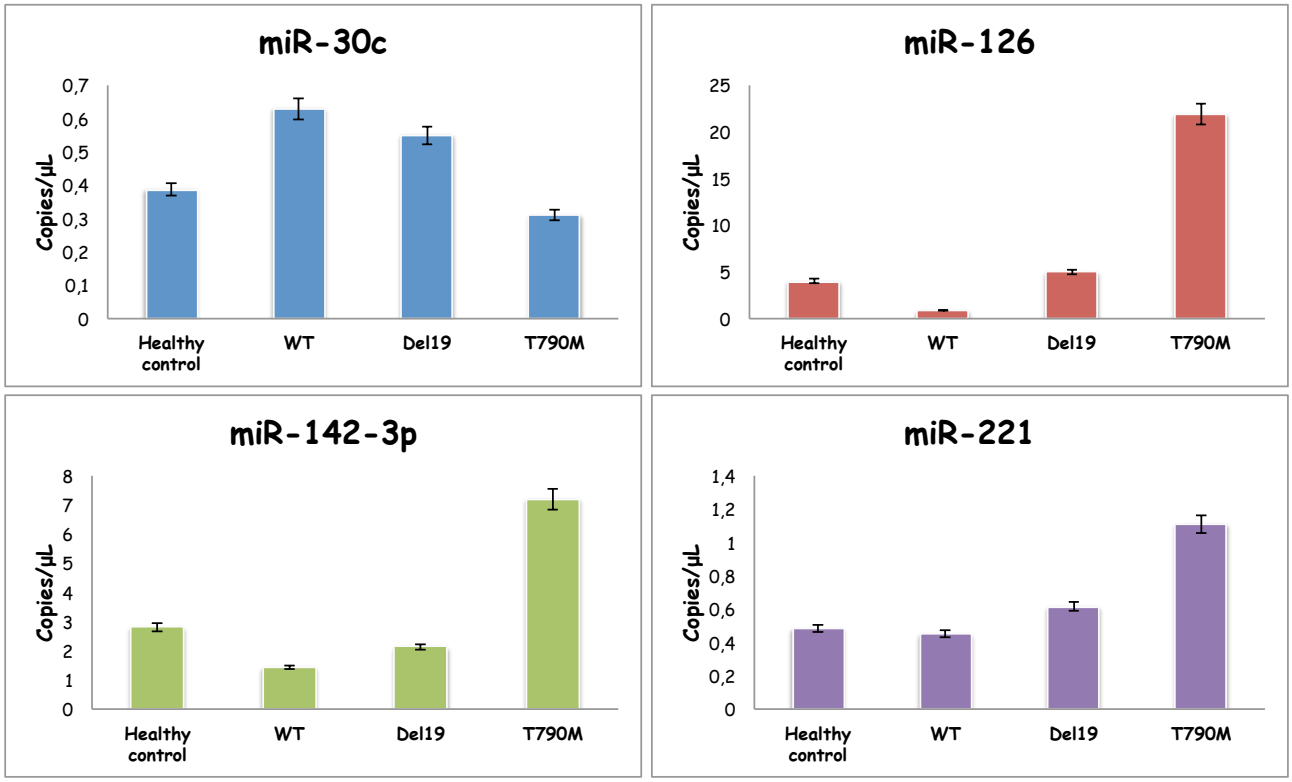

Figure 18: bar graph showing copies/µL of circulating miR-30c, miR-126, miR-142-3p, miR-221 in healthy control, wild type, Del19 and T790M selected patients.

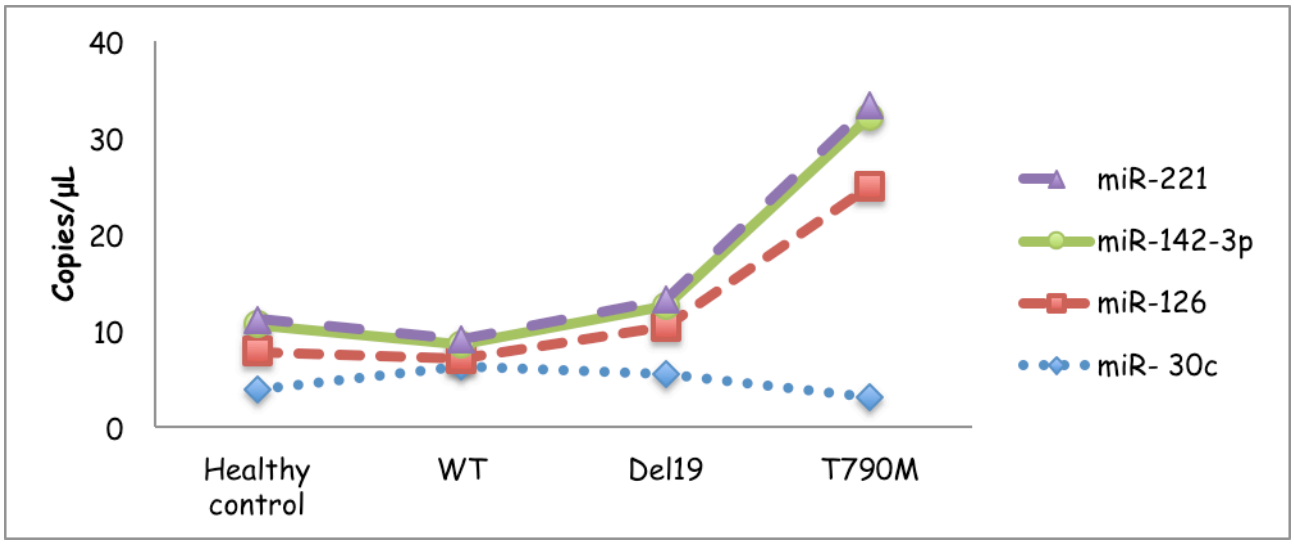

Figure 19: chart showing the cumulative trend of circulating miR-30c, miR-126, miR-142-3p, miR-221 along EGFR molecular status in NSCLC.

Patient #	Sex	Histotype	EGFR mutation
#13	M	Adenocarcinoma	wild type
#15	F	Adenocarcinoma	wild type
#19	M	Adenocarcinoma	wild type
#27	F	Adenocarcinoma	Del19
#47	F	Adenocarcinoma	Del19
#51	M	Adenocarcinoma	Del19
#14	M	Adenocarcinoma	Del19+T790M
#16	F	Adenocarcinoma	Del19+T790M
#25	M	Adenocarcinoma	Del19+T790M
Healthy C	M	Adenocarcinoma	NA
Healthy C	M	Adenocarcinoma	NA
Healthy C	F	Adenocarcinoma	NA

Table 1: this table resumes the main characteristics of patients involved in miRNAs analysis

Patient #	Gender	Histotype	EGFR tissue	EGFR plasma	[ng/mL]
#1	F	adenocarcinoma	Del19	T790M	910
#2	F	adenocarcinoma	Del19	Del19+T790M	430
#3	F	adenocarcinoma	Del19	Not found	520
#4	M	adenocarcinoma	Del19	Del19+T790M	1030
#5	F	adenocarcinoma	Del19	Del19	290
#7	M	adenocarcinoma	Del19	Del19	1000
#10	F	adenocarcinoma	Del19	T790M	560
#11	F	adenocarcinoma	Del19	T790M	800
#12	F	adenocarcinoma	L858R	L858R	2920
#14	M	adenocarcinoma	Del19	T790M	430
#16	F	adenocarcinoma	Del19	Del19+T790M	8000
#17	M	adenocarcinoma	L858R	L858R+T790M	500
#18	M	adenocarcinoma	Ins20	Not found	400
#21	M	adenocarcinoma	Del19	Not found	840
#22	F	adenocarcinoma	L858R	Not found	630
#23	M	adenocarcinoma	Del19	Del19+T790M	7930
#25	M	adenocarcinoma	Del19	T790M	700
#26	F	adenocarcinoma	Del19	Not found	13100
#27	F	adenocarcinoma	Del19	Not found	400
#29	F	adenocarcinoma	Del19	Del19+T790M	670
#34	F	adenocarcinoma	Del19	Not found	860
#35	F	adenocarcinoma	Del19	Del19+T790M	2840
#41	F	adenocarcinoma	L858R	Not found	770
#42	M	adenocarcinoma	L858R	L858R+T790M	760
#44	F	adenocarcinoma	Del19	Not found	520
#47	F	adenocarcinoma	Del19	Del19	600

#48	F	adenocarcinoma	L858R	L858R	1720
#50	F	adenocarcinoma	L858R	Not found	580
#51	M	adenocarcinoma	Del19	Del19	970
#53	F	adenocarcinoma	L858R	Not found	320
#56	F	adenocarcinoma	Del19	Del19+T790M	350
#57	M	adenocarcinoma	Del19	Del19	200
#59	M	adenocarcinoma	Del19	Del19+T790M	590
#60	M	adenocarcinoma	Del19	Del19+T790M	520

Table 2: this table resumes the main characteristics of patients enrolled in the PD sub-cohort.

Patient #	Gender	Histotype	EGFR plasma	[ng/mL]
#6	M	adenocarcinoma	Not found	700
#8	M	adenocarcinoma	Not found	500
#9	M	adenocarcinoma	Not found	700
#13	M	adenocarcinoma	Not found	430
#15	F	adenocarcinoma	Not found	600
#19	M	adenocarcinoma	Not found	530
#20	M	adenocarcinoma	Del19	300
#24	M	adenocarcinoma	Not found	700
#28	M	adenocarcinoma	Not found	1200
#30	M	adenocarcinoma	Not found	680
#31	F	adenocarcinoma	Not found	970
#32	M	adenocarcinoma	Not found	620
#33	F	adenocarcinoma	Not found	400
#36	M	adenocarcinoma	Not found	860
#37	F	adenocarcinoma	Not found	1810
#38	M	adenocarcinoma	L858R	1060
#39	M	adenocarcinoma	Del19	1600
#40	F	adenocarcinoma	Not found	990
#43	F	adenocarcinoma	Not found	890
#45	M	adenocarcinoma	Not found	630
#46	M	adenocarcinoma	Not found	450
#49	M	adenocarcinoma	Not found	830
#52	F	adenocarcinoma	L858R+T790M	480
#54	M	adenocarcinoma	Not found	230
#55	F	adenocarcinoma	Not found	300
#58	M	adenocarcinoma	Not found	610

Table 3: this table resumes the main characteristics of patients enrolled in the D sub-cohort.

PATIENTS' CHARACTERISTICS		Median cfDNA (baseline)	Median cfDNA (follow-up)
Age	>70 years: 14 (34%)	0,55 ng/µl	0,65 ng/µl
	<70 years: 27 (66%)	0,43 ng/µl	0,52 ng/µl
Gender	Male: 29 (70%)	0,49 ng/µl	0,54 ng/µl
	Female: 12 (30%)	0,43 ng/µl	0,63 ng/µl
Smoking Status	Never: 7 (17%)	0,47 ng/µl	0,5 ng/µl
	Current/Former: 34 (83%)	0,47 ng/µl	0,39 ng/µl
Histology	Squamous: 15 (35%)	0,55 ng/µl	0,61 ng/µl
	Non-squamous: 26 (65%)	0,49 ng/µl	0,49 ng/µl
Prior lines of therapy	1: 18 (44%)	0,43 ng/µl	0,44 ng/µl
	>2: 23 (56%)	0,46 ng/µl	0,62 ng/µl
Best responce (RECIST)	CR+PR+SD: 21 (51%)	0,36 ng/µl	0,35 ng/µl
	PD: 20 (49%)	0,58 ng/µl	0,79 ng/µl
Overall survival	Median OS: 9.9 months		
	OS > 6 months: 24 (53%)	0,38 ng/µl	0,39 ng/µl
	OS < 6 months: 17 (47%)	0,58 ng/µl	0,77 ng/µl

Table 4: this table resumes the characteristics of patients enrolled in the study of cfDNA as surrogate biomarker, at baseline or follow-up, during immunotherapy administration.

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