UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ESCOLA TÈCNICA SUPERIOR D´ENGINYERIA AGRONÒMICA I DEL MEDI NATURAL

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

TRABAJO FIN DE GRADO DE BIOTECNOLOGÍA

Applicability of digital PCR at diagnosis and monitoring of EGFR-mutated patients in advanced Non-Small Cell Lung Cancer.

ALUMNA: MARÍA FERNÁNDEZ FERNÁNDEZ
TUTORES: ELOISA JANTUS LEWINTRE Y SILVIA CALABUIG FARIÑAS

Curso Académico: 2018-2019

VALENCIA, 8 de julio de 2019

TITLE: Applicability of digital PCR at diagnosis and monitoring of EGFR-mutated patients in advanced Non-Small Cell Lung Cancer**.**

SUMMARY:

- **BACKGROUND:** Currently, lung cancer is the leading cause of cancer incidence and mortality worldwide for both sexes. Non-Small Cell Lung Cancer (NSCLC) represents the 85% of all lung cancer types. Treatment and cancer prognosis are closely related to the type and stage of the tumor identified. Therefore, the discovery of somatic driver mutations in epidermal growth factor receptor gene (*EGFR*), which occurs in approximately 10-30% of NSCLC patients, set the stage for science-based precision medicine in the management of advanced NSCLC. Since then, research has achieved many milestones that have transformed the clinical management of this disease. Targeted treatments with *EGFR* tyrosine kinase inhibitors (TKIs) resulted in improved outcomes with less toxicity compared to standard chemotherapy. Nevertheless, acquired resistance to EGFR-TKIs may appear in almost all patients after 10-14 months of treatment. The most common mechanism of resistance is the acquired T790M mutation in exon 20. In this context, real-time monitoring of *EGFR* mutations is essential to determine the most appropriate therapeutic decisions for each patient. In the last years, liquid biopsy has arrived as a first option to early detect resistance mechanisms demonstrating its potential as a minimally invasive technique to get relevant tumor information.

- **METHODS**: Consecutive patients with advanced *EGFR*-mutant NSCLC and treated with TKIs were enrolled in this study. Plasma samples were collected at the time of diagnosis and throughout standard TKI treatment until progression. Circulating tumor DNA (ctDNA) was isolated and analyzed to investigate *EGFR* mutational status by two different digital PCR approaches. The first approach, BEAMing (Sysmex®), is based on emulsion PCR, where templates are clonally amplified using magnetic beads. The second one, called Quant Studio™ uses microwells to split the sample into thousands of partitions. Results from both methods were compared and correlated with clinical variables.

- **RESULTS:** Of 18 patients analyzed, the concordance ratio between tissue and plasma was 61.1 %. In 80% of the patients with progressive disease, the T790M mutations was detected as resistance mechanism in plasma. Good correlation between the two digital PCR methods evaluated was found.

- **CONCLUSION**: The results presented in this work suggest that dPCR technique for the analysis of ctDNA in advanced NSCLC is a sensitive technology for *EGFR* mutational status analysis. In this context, it represents the beginning of an innovative approach to molecular diagnostics of cancer, which has the potential to inform early detection of cancer, detect minimal residual disease, mirror the heterogeneity of tumor and track evolution of resistant disease.

- **KEY WORDS**: Non-small-cell lung cancer (NSCLC); circulating tumor DNA (ctDNA); liquid biopsy; epidermal growth factor receptor mutations (*EGFR* mutations); digital PCR (dPCR).

Author: María Fernández Fernández
Location and date: Valencia, July 2019
Academic supervisor: Eloisa Jantus Lewintre
Institution supervisor: Silvia Calabuig Fariñas

TÍTULO: Aplicabilidad de la PCR digital en el diagnóstico y monitorización de pacientes *EGFR* mutados en cáncer de pulmón no microcítico (CPNM) avanzado.

RESUMEN:

- **ANTECEDENTES**: Actualmente, el cáncer de pulmón es la principal causa de incidencia y mortalidad de cáncer en todo el mundo para ambos sexos. El cáncer de pulmón no microcítico (CPNM) representa el 85% de todos los tipos de este cáncer. El tratamiento y el pronóstico del cáncer están estrechamente relacionados con el tipo y el estadio del tumor. Por lo tanto, el descubrimiento de mutaciones somáticas en el gen del receptor del factor de crecimiento epidérmico (*EGFR*), que ocurre en aproximadamente el 10-30% de los pacientes con CPNM, supuso una mejora significativa en el manejo clínico de los pacientes con CPCNP avanzado. Los tratamientos dirigidos con inhibidores de la tirosina quinasa de *EGFR* (TKI, por sus siglas en inglés) dieron mejores resultados con menos toxicidad en comparación con la quimioterapia estándar. Sin embargo, la resistencia adquirida a EGFR-TKIs puede aparecer en casi todos los pacientes después de 10-14 meses de tratamiento. El mecanismo de resistencia más común es la adquisición de la mutación T790M en el exón 20 del gen de *EGFR*. En este contexto, la monitorización en tiempo real de las mutaciones de *EGFR* es esencial para determinar las decisiones terapéuticas más apropiadas para cada paciente. En los últimos años, la biopsia líquida ha llegado como una primera opción para detectar precozmente mecanismos de resistencia siendo esta mínimamente invasiva para obtener información relevante sobre el tumor.

- **MÉTODOS:** En este estudio se incluyeron pacientes con CPNM avanzado, mutados en *EGFR* y tratados con TKI. Las muestras de plasma se recolectaron en el momento del diagnóstico y durante todo el tratamiento estándar con TKI hasta la progresión. El ADN tumoral circulante (ctDNA) se aisló y analizó para investigar el estado mutacional de *EGFR* mediante dos enfoques de PCR digital diferentes. La primera aproximación, BEAMing (Sysmex®), se basa en una PCR de emulsión, donde las moléculas de ADN diana se amplifican por clonación utilizando partículas magnéticas. La segunda, denominada Quant Studio™, usa micropocillos para dividir la muestra en miles de particiones. Los resultados de ambos métodos fueron comparados y correlacionados con variables clínicas.

- **RESULTADOS**: De los 18 pacientes analizados, la relación de concordancia entre tejido y plasma fue del 61.1%. En el 80% de los pacientes con enfermedad progresiva, la mutación T790M se detectó como mecanismo de resistencia en ctDNA. Se encontró buena correlación entre los dos métodos de PCR digital evaluados.

- **CONCLUSIÓN**: Los resultados presentados en este trabajo sugieren que la técnica de dPCR para el análisis de ctDNA en CPNM avanzado es una tecnología sensible para el análisis del estado mutacional de *EGFR*. En este contexto, representa el comienzo de un enfoque innovador para el diagnóstico molecular del cáncer, que tiene el potencial de informar la detección temprana del cáncer, detectar la enfermedad residual mínima, reflejar la heterogeneidad del tumor y controlar la evolución de la enfermedad resistente.

PALABRAS CLAVE: Cáncer de pulmón no microcítico (CPNM); ADN tumoral circulante (ctDNA); biopsia líquida; mutaciones del receptor del factor de crecimiento epidérmico (mutaciones *EGFR*); PCR digital (dPCR).

Autor/a: María Fernández Fernández
Localización y fecha: Valencia, Julio 2019
Tutor/a académico: Eloisa Jantus Lewintre
Tutor/a institución: Silvia Calbuig Fariñas

TÍTOL: Aplicabilitat de la PCR digital en el diagnostic y monitorització de pacients *EGFR* mutats en Càncer de Pulmó no Microcític (CPNM) avanzat.

RESUM:

- **ANTECEDENTS**: Actualment, el càncer de pulmó és la principal causa d'incidència i mortalitat de càncer a tot el món per a tots dos sexes. El càncer de pulmó no microcític (CPNM) representa el 85% de tots els tipus d'aquest càncer. El tractament i el pronòstic del càncer estan estretament relacionats amb el tipus i l'estadi del tumor. Per tant, el descobriment de mutacions somàtiques en el gen del receptor del factor de creixement epidèrmic *(EGFR),* que ocorre en aproximadament el 10-30% dels pacients amb CPNM, va suposar una millora significativa en el maneig clínic dels pacients amb CPNM avançat. Els tractaments dirigits amb inhibidors de la tirosina quinasa de *EGFR* (TKI, per les sigles en anglès) van donar millors resultats amb menys toxicitat en comparació amb la quimioteràpia estàndard. No obstant això, la resistència adquirida a EGFR-TKIs pot aparèixer en gairebé tots els pacients després de 10-14 mesos de tractament. El mecanisme de resistència més comú és l'adquisició de la mutació T790M en l'exó 20 del gen d'*EGFR*. En aquest context, la monitorització en temps real de les mutacions d'*EGFR* és essencial per determinar les decisions terapèutiques més apropiades per a cada pacient. En els últims anys, la biòpsia líquida ha arribat com una primera opció per detectar precoçment mecanismes de resistència sent aquesta mínimament invasiva per obtenir informació rellevant sobre el tumor.

- **MÈTODES**: En aquest estudi es van incloure pacients amb CPNM avançat, mutats en *EGFR* i tractats amb TKIs. Les mostres de plasma es van recol·lectar en el moment del diagnòstic i durant tot el tractament estàndard amb TKI fins a la progressió. L'ADN tumoral circulant (ctDNA) es va aïllar i analitzar per investigar l'estat mutacional d'*EGFR* mitjançant dos mètodes de PCR digital diferents. El primer mètode, Sysmex® Beaming, es basa en la PCR d'emulsió, on les plantilles s'amplifiquen per clonació utilitzant partícules magnètiques. El segon, anomenat Quant Studio™ fa servir micropouets per dividir la mostra en milers de particions. Els resultats d'ambdós mètodes es van comparar y relacionar amb variables clíniques.

- **RESULTATS:** Dels 18 pacients analitzats, la relació de concordança entre teixit i plasma va ser del 61.1%. En el 80% dels pacients amb malaltia progressiva, les mutacions T790M es van detectar com a mecanisme de resistència en plasma. Es va trobar bona correlació entre els dos mètodes de PCR digital avaluats.

- **CONCLUSIÓ**: Els resultats presentats en aquest treball suggereixen que la tècnica de dPCR per l'anàlisi de ctDNA en CPNM avançat és una tecnologia sensible per l'anàlisi de l'estat mutacional d'*EGFR*. En aquest context, representa l'inici d'un enfocament innovador per al diagnòstic molecular del càncer, que té el potencial d'informar la detecció primaria del càncer, detectar la malaltia residual mínima, reflectir l'heterogeneïtat del tumor i controlar l'evolució de la malaltia resistent.

PARAULES CLAU: Càncer de pulmó no microcític (CPNM); ADN tumoral circulant (ctDNA); biòpsia líquida; mutacions del receptor del factor de creixement epidèrmic (mutacions *EGFR*); PCR digital (dPCR).

Autor/a: María Fernández Fernández
Localització i data: Valencia, juliol 2019
Tutor/a académic: Eloisa Jantus Lewintre
Tutor/a institució: Silvia Calabuig Fariñas

INDEX

FIGURE INDEX

TABLE INDEX

ABBREVIATIONS

ADC: Adenocarcinoma.

ALK: Anaplastic Lymphoma Kinase.

ARMS: Amplification Refractory Mutation System.

ATP: Adenosine triphosphate.

BEAM: Beads, Emulsion, Amplification, Magnetics.

bFGF: basic Fibroblast Growth Factor.

cDNA: complementary DNA.

cfDNA: circulating free DNA.

CSGE: Conformation-Sensitive Gel Electrophoresis.

Ct: Cycle threshold.

CT-scan: Computed tomography scan.

ctDNA: circulating tumor DNA.

CTCs: Circulating Tumor Cells.

Del: deletion.

dPCR: Digital Polymerase Chain Reaction.

EDTA: Ethylenediamine tetraacetic acid.

***EGFR*:** Epidermal Growth Factor Receptor.

emPCR: Emulsion PCR.

ESMO: European Society for Medical Oncology.

FDA: Food and Drug Administration.

IASLC: International Association for the Study of Lung Cancer.

IVD: In Vitro Diagnostic.

kDa: kilo Daltons.

LB: Liquid Biopsy.

LCC: Large Cell Carcinoma.

LoD: Limit of Detection.

MAF: Mutant Allelic Fraction.

mPCR: multiplex PCR.

NSCLC: Non-small Cell Lung Cancer.

PCR: Polymerase Chain Reaction.

qPCR: quantitative PCR.

SEOM: Spanish Society of Medical Oncology.

SCLC: small Cell Lung Cancer.

SCC: Squamous Cell Carcinoma.

TKIs: Tyrosine Kinase Inhibitors.

TLS: Tertiary Lymphoid Structures.

TM: Transmembrane Protein.

VEGFR: Vascular Endothelial Growth Factor Receptor.

WT: Wild-type.

1. INTRODUCTION

1.1. THE CONCEPT OF CANCER

Cancer is a complex disease characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs (World Health Organization, 2018). It can affect almost any part of the body and has many anatomic and molecular subtypes that each require specific management strategies.

Cancer incidence and mortality are rapidly growing worldwide, being the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The reasons are complex, but aging and growth of the population are the most important, as well as changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development (Jemal et al, 2011).

1.1.1. Molecular Biology of Cancer

The mechanism of cancer tumorigenesis is dependent on the reprogramming of cellular metabolism as both direct and indirect consequence of oncogenic mutations. The alterations in intracellular and extracellular metabolites that can accompany cancer-associated metabolic reprogramming, have important effects on gene expression, cellular differentiation, and the tumor microenvironment (Pavlova and Thompson, 2016).

Moreover, tumor cells subsist in a rich microenvironment provided by resident fibroblasts, endothelial cells, pericytes, leukocytes, and extra-cellular matrix (Pietras & Östman, 2010) together with the immune contexture that can have different effects on tumor progression (Figure 1) (Fridman et al., 2012). During the course of tumor progression, the interactions with the host create a tissue microenvironment which is comprised of proliferating tumor cells, the tumor stroma, blood vessels, Infiltrating inflammatory cells and a variety of associated tissue cells (TL, 2008).

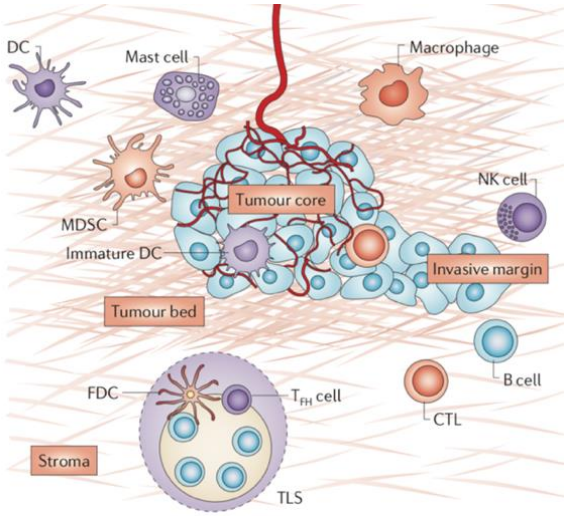


Figure 1. The immune contexture. Tumor anatomy showing the features of the immune contexture, including the tumor core, the invasive margin, Tertiary Lymphoid Structures (TLS) and the tumor microenvironment (Fridman et al., 2012).

Collaborative interactions between neoplastic cancer cells and their supporting stroma contribute to form local invasions, metastases, or vascular niches for hematopoietic malignancies (Hanahan and Coussens, 2012). Cancer cells accumulate metabolic alterations that allow them to gain access to nutrient sources and use them to create new biomass to sustain deregulated proliferation, and take advantage of the ability of certain metabolites to affect the proliferation of cancer cells themselves as well as a variety of normal cell types within the tumor microenvironment (Pavlova and Thompson, 2016).

In order to study and identify the complex tumorigenic mechanisms, they have been categorized into ten characteristics, also known as "The hallmarks of cancer", comprising the main biological capabilities acquired during the multistep development of human tumors. They include sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor promoting inflammation, activating invasion and metastasis, inducing neovascularization, genome instability and mutation, resisting cell death and deregulating cellular energetics (Figure 2) (Hanahan and Weinberg, 2011).

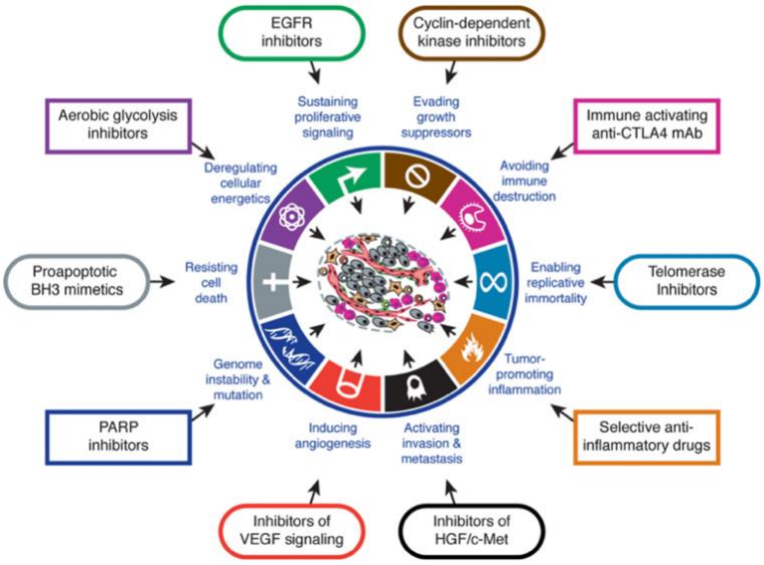


Figure 2. The ten hallmarks of cancer and their therapeutic approach (Hanahan & Weinberg, 2011).

1.2. LUNG CANCER

1.2.1. Epidemiology

Lung cancer is the leading cause of cancer incidence and mortality worldwide for both sexes, with 2.1 million new lung cancer cases and 1.8 million deaths predicted in 2018, representing close to 1 in 5 (18.4%) cancer deaths (Figure 3)(Bray, GLOBOCAN, 2018).

Nowadays the incidence of cancer in Spain is around 250.000 new cases every year, being lung cancer approximately the 28.65% of the diagnosed tumors. In addition, cancer is the second cause of death in Spain and the responsible of the largest number of deaths related to cancer, causing a total of 22.481 deaths in 2018 (SEOM, 2018).

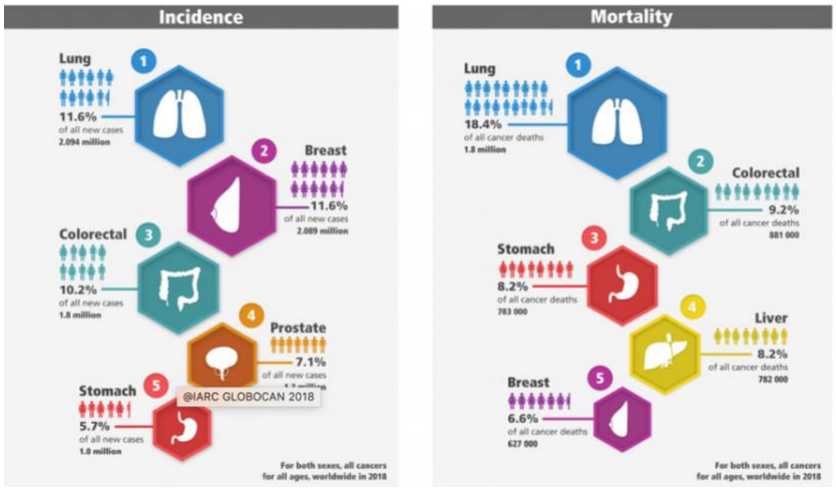


Figure 3. Percentages of new cancer cases and cancer deaths worldwide in 2018 (GLOBOCAN, 2018).

1.2.2. Risk factors

The most important cause of lung cancer is exposure to tobacco smoke through active or passive smoking (Hwang et al., 2003, Heuvers et al., 2012).

There are other risk factors such as outdoor air pollution, radiation exposure and dietary constituents. Among these, occupational exposures due to asbestos, silica, radon, diesel engine exhausts and mineral oils should be taken into account, as they have been associated to a 15% of lung cancer cases in the UK (Parkin, 2011). Nevertheless, there is still a lack of explanation for lung cancer in non-smokers that could also be due to history of lung disease or early-onset cancer and genetic factors (Brenner et al., 2010).

1.2.3. Diagnosis and prognosis

There are some symptoms related to the risk factors explained before that allow early lung cancer detection, such as chronic cough, dyspnea, hemoptysis, weight loss, and chest pain (Bezjak et al., 2006). But, in more than 70% of the cases, lung cancer diagnosis occurs at advanced stages of the disease when curative intervention such as surgery is no longer possible (Jantus-Lewintre et al., 2012). The initial evaluation of patients relies on patient history and physical examination, laboratory tests, imaging techniques and a confirmatory biopsy, that allows the acquisition of tissue samples for pathological analysis Nevertheless, most of times this procedure is very difficult and the amount of tissue obtained is not enough for molecular diagnosis of the tumor (Collins et al., 2007).

Treatment and prognosis are closely related to the histological subtypes and tumor stage (Detterbeck et al., 2003), which is based on the TNM classification relaying in the size and degree of locoregional invasion by the primary tumor (T), the extend of regional lymph node involvement (N) and the presence or absence of intrathoracic or distant metastases (M) (Shepherd et al., 2007, Mirsadraee et al., 2012). Afterwards, molecular diagnostic techniques are applied to detect targetable oncogenic alterations or immune-related biomarkers (Planchard et al., 2018).

1.2.4. Pathology classification

Lung cancer comprises a very heterogeneous collection of neoplasia. To facilitate treatment and prognostic decisions, lung cancer has been histologically classified in two main groups: the non–small cell carcinoma (NSCLC) and the small cell carcinoma (SCLC), representing an 85-90% and 10-15%, respectively (Collins et al., 2007).

NSCLC is subclassified according to histology into three main subtypes, squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell carcinoma (LCC) (Srivastava et al., 2010, Chen et al., 2017). SCC arises mainly from the central airways and it is more related with males and smoking habits, while ADC is peripherally located and represents 40% of all NSCLCs and LCC represents less differentiated forms of this type of tumors (Barberis et al., 2016).

1.2.5. Treatment

Nowadays, surgical resection is the treatment of choice for patients with stage I to IIIA (Crinò et al., 2010). For many years, chemotherapy with a platinum-doublet has remained the gold standard for NSCLC treatment at advanced stages of the disease, although currently there are different therapeutic strategies available.

On one hand, immunotherapy has achieved a remarkable improvement in patient prognosis by stimulating patient's immune system to exert more efficient anti-tumor responses by blocking immune-checkpoints such as PD-1 or PD-L1 (Topalian et al., 2016; Planchard et al., 2018).

On the other hand, there are many targeted therapies such as tyrosine kinase inhibitors (TKIs) directed towards specific mutations on *EGFR* or *ALK* genes, among others. Current therapeutic strategies directed to *EGFR* mutated tumors are explained in detail later. The role of such specific mutations in the pathogenesis of lung cancers is still unclear (Shigematsu et al., 2005) and more research needs to be done in order to elucidate the remaining mechanisms of resistance. The successful and appropriate translation of cancer genome research into clinical practice will raise important social and ethical questions (International & Genome, 2010).

1.3. *EGFR* ROLE IN TUMORIGENESIS

1.3.1. *EGFR* structure and function

The epidermal growth factor receptor (*EGFR*) is a transmembrane glycoprotein (170 kDa) with an extracellular ligand binding domain and an intracellular tyrosine kinase (TK) domain that regulate signaling pathways to support proliferation and survival of cancer cells (Yun et al., 2007). The human *EGFR* gene is located in the short arm of human chromosome 7 (7p12), and it comprises 28 exons and 27 introns. Exons 1 to 16 encode the extracellular domain, while exon 17 codes for the transmembrane domain, and exons 18 to 28 the intracellular ones. Within this last part, the TK domain is encoded by exons 18 to 24 and the C-terminal domain is encoded by exons 25 to 28 (Inamura et al., 2010).

EGFR belongs to the erbB family of closely related receptor tyrosine kinases, which include erbB1 (also known as *EGFR*), erbB2 (*HER2*), erbB3, and erbB4. Although their basic structures are similar, each one has distinct properties, including variation in its tyrosine kinase activity (Bethune et al., 2010).

Generally, aberrant *EGFR* signaling, activates three major pathways (Figure 4), the mitogen-activated protein kinase (MAPK) pathway, the signal transducers and activator of transcription (STAT) pathway, and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Inamura et al., 2010).

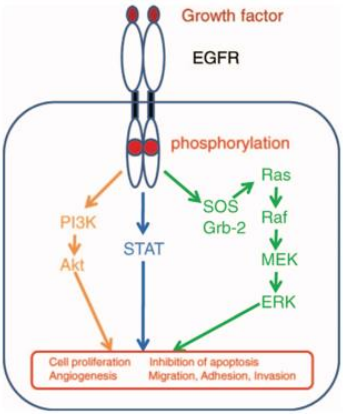


Figure 4. Schematic illustration of the *EGFR* and downstream signaling pathways. Binding of a receptor-specific ligand leads to phosphorylation of *EGFR* and signaling through the mitogen-activated protein kinase (MAPK) pathway (green), signal transducers and activator of transcription (STAT) pathway (blue), and phosphatidylinositol-3-kinase (PI3K)/Akt pathway (orange). These pathways promote cell proliferation, neovascularization, migration, adhesion, and/or invasion, while inhibiting apoptosis. Other proteins involved are son of sevenless (SOS), growth factor receptor–bound protein 2 (Grb- 2), oncoproteins Ras and Raf, mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) (Inamura et al., 2010).

1.3.2. Most common *EGFR* mutations in lung cancer

EGFR-mutant NSCLC cells are dependent on the aberrant kinase signaling for survival. All of the somatic activating *EGFR* mutations involve the adenosine triphosphate (ATP)-binding pocket in the receptor of the TK domain. Kinase domain mutations in *EGFR* are referred to as 'activating mutations' because they lead to a ligand-independent activation of TK activity.

EGFR tyrosine kinase (TK) domain mutations are the first molecular change known to occur specifically in never smokers, which are no cause by tobacco carcinogens (Kosaka et al., 2004, Shigematsu et al., 2005). These mutations occur within *EGFR* exons 18–21, which encode a portion of the *EGFR* kinase domain, and are a prime example of the complexity of the disease at the molecular level (Figure 5). Mutations involving exons 18, 19, and 21 are considered predictive of sensitivity to *EGFR* tyrosine kinase inhibitors (TKIs), which are known as 'sensitizing' mutations (Cardenal et al., 2009, Uchida et al., 2015).

The two most common sensitizing *EGFR* gene mutations are L858R and exon 19 deletion (exon 19 del). The L858R mutation within exon 21 is observed in approximately 43% of *EGFR* mutation-positive NSCLCs, and it comprises a leucine–to–arginine substitution at position 858 in EGFR protein. In contrast, *EGFR* exon 19 del is an in-frame deletion occurring within exon 19 and is found in approximately 48% of EGF*R*-mutated lung tumors (Mitsudomi & Yatabe, 2010). Another less common mutation, *EGFR* exon 20 insertion, is seen in 4% to 9% of *EGFR* mutation-positive NSCLC (Yang et al., 2015).

On the other hand, there are also secondary mutations that usually develop as resistance to EGFR-TKIs treatment. The T790M mutation is a second-site mutation which involves a threonine-to-methionine substitution in exon 20 and it has been detected in approximately 50% to 60% of patients in whom acquired resistance to EGFR-TKIs develops (Yun et al., 2008).

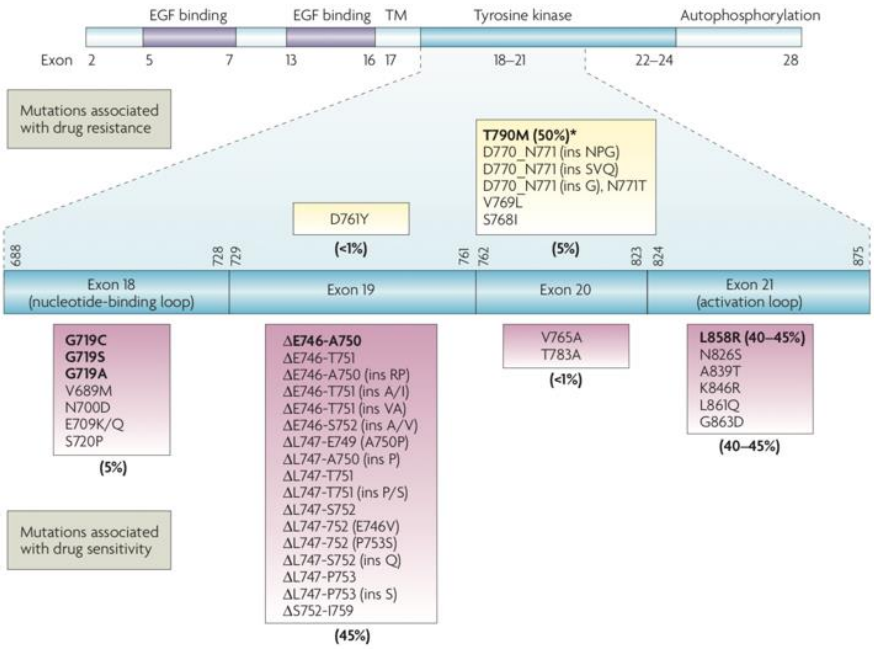


Figure 5. Most common *EGFR* mutations in NSCLC. Representation of the epidermal growth factor receptor (*EGFR*) gene showing the distribution of exons in the extracellular domain (EGF binding domain), transmembrane domain (TM) and intracellular domain (comprising the tyrosine kinase and autophosphorylation regions) (Sharma et al., 2007).

1.3.3. EGFR-targeted therapies

Mutations on *EGFR* results in alteration of affinity of *EGFR* tyrosine kinase to ATP and, consequently, *EGFR* and downstream signaling pathways alteration. This is due to the three-dimensional structural alterations in the *EGFR* tyrosine kinase domain. For that reason, inhibition of this bound with EGFR-TKIs, results in cell death that is mediated through the intrinsic apoptotic pathway (Pao, William; Chmielecki, 2010).

Although patients with specific *EGFR* gene-activating mutations are more likely to have amplified *EGFR* or high *EGFR* expression, it is the mutation status alone that predicts response to EGFR-TKIs in first-line therapy (Gately et al., 2012).

First-generation EGFR-TKIs approved by the FDA, gefitinib and erlotinib, are reversible competitive inhibitors of the tyrosine kinase domain of *EGFR* that bind to its adenosine-5' triphosphate-binding site in order to compete with ATP molecules in the TK domain (Gadzar, 2010). Using this blockage mechanism, gefitinib and erlotinib induce apoptosis and inhibit growth and cell proliferation (Gately et al., 2012).

These first-generation EGFR-TKIs improve progression-free survival when compared to chemotherapy in patients with EGFR-activating mutations in the first-line setting. However, nearly all patients develop resistance to EGFR-directed agents and new therapeutic options should be considered. Afatinib is a second-generation EGFR-TKI defined as an ErbB family blocker that inhibits *EGFR* TK domain and, in contrast with first-generation EGFR-TKIs, it is capable to bind irreversibly to the TK domain of the *EGFR* gene (Nelson et al., 2013).

Eventually, patients with sensitizing *EGFR* mutations, can develop resistance to first- and second-generation EGFR-TKIs by the acquisition of secondary mutations, such as T790M which increases the affinity of mutant *EGFR* for ATP. Osimertinib is a third-generation EGFR-TKI that targets both sensitizing *EGFR* mutations and the resistant exon T790M mutation (Planchard et al., 2018). Therefore, osimertinib represents the new standard of care in the treatment of T790M-positive NSCLC patients resistant to previous generation EGFR-TKIs (La Monica et al., 2017).

1.4. LIQUID BIOPSY

As it was mentioned before, molecular diagnosis in cancer certainly requires the analysis of a tumor biopsy. However, in lung cancer, there is still a 20- 30% of tissue failure rates for tumor genotyping in routine pathological samples. As a consequence, liquid biopsy (LB) has emerged as a valid alternative source of information for the analysis of tumor specific alterations. LB refers to specimens obtained from body fluid such as blood, urine, saliva and cerebrospinal fluid, among others.

Without any doubt, blood is the most explored LB samples. The main clinical developments in peripheral blood have focused on the analysis of: i) circulating tumor DNA (ctDNA), which represents a small part of cell free circulating DNA released from tumor cells and, therefore, carries mutations or other genomic/epigenomic tumor alterations and ii) circulating tumor cells (CTCs), defined as disseminated cancer cells in the bloodstream. Each of these materials offers unique opportunities to test different biomarkers and to analyze tumor features (Calabuig-Fariñas et al., 2016).

The advantages of the use of blood samples are clear: i) it is a minimally invasive way to get relevant tumor information, ii) serial samples can be obtained capturing tumor evolution in real time, iii) LB abrogates the limitations associated with tumor heterogeneity, since nucleic acids or tumor cells present in circulation recapitulate the information belonging from different tumor locations (primary tumor and metastases), iv) the development of new sensitive assays for analyses of ctDNA and CTCs allows the assessment of minimal residual disease and v) the costs of LB analysis are comparable with other molecular biology techniques already used in the clinical setting in addition to the reduced risks of complications associated with tissue biopsy (Figure 6).

In the context of lung cancer with molecular targets, LB can represent a key opportunity in the implementation of precision oncology, allowing a real-time monitoring of targeted therapies mainly through the analysis of ctDNA (Alix-Panabieres et al., 2012). As it was stated previously, most of the tumors treated with EGFR-TKIs, acquire resistance as a result of clonal evolution and selection. Therefore, LB is an extremely useful tool to early-detect resistances (Bartels et al., 2017).

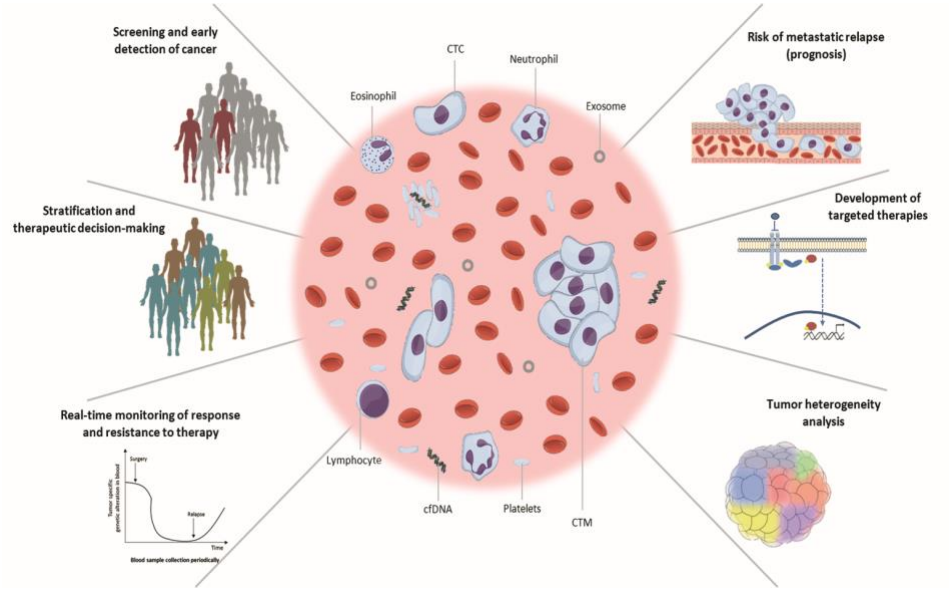


Figure 6. The potential clinical benefits of liquid biopsy (Calabuig-Fariñas et al., 2016)**.**

1.4.1. ctDNA

Tumor DNA can be released into the blood of a patient with cancer in form of ctDNA. The majority of such ctDNA is derived from apoptotic and necrotic tumor cells from primary tumors, CTCs, micrometastases, or metastases (Alix-Panabieres et al., 2012). Two mechanisms have been described for ctDNA releasing into the bloodstream, the passive and the active mechanisms. In the first one, the ctDNA is released directly from apoptotic and necrotic tumor cells or indirectly by necrotic tumor cells engulfed by macrophages. On the other hand, the active release may be due to the association of ctDNA with a protein complex to act as an intercellular messenger (Stroun et al., 2006).

It is possible that mutational signatures in ctDNA could distinguish clinically insignificant biological processes from malignant and lethal biological processes. In addition, serial measurements of the ctDNA signal may identify distinct trajectories with different kinetics for indolent versus lethal disease (Aravanis et al., 2017). Analysis of ctDNA in plasma samples obtained before and after treatment can ultimately provide a global picture of the genetic alterations of a patients tumor (Diaz & Bardelli, 2014). Moreover, it has been showed that ctDNA levels are higher in diseased than in healthy individuals (Crowley et al., 2013, Qi et al., 2018) indicating that it is possible to analyse minimal residue after tumor removal.

In cancer diagnosis and treatment, detection of ctDNA derived from tumors, has been challenging for three primary reasons, which include: presence of sometimes extremely low levels of ctDNA, discrimination of ctDNA from normal circulating free DNA (cfDNA); and the accurate quantification of the number of mutant fragments in a sample.

Another potential application of ctDNA is the detection of minimal residual disease after surgery or therapy with curative intentions. In particular, the *EGFR* mutational status can be assessed on ctDNA, even if it only represents a small fraction (<0.5%) of the cfDNA released into the blood. Most clinical trials adopted real-time PCR (RT-PCR) or digital PCR (dPCR) to compare the performance of *EGFR* testing in cfDNA versus tumor tissue (Malapelle et al., 2016).

Monitoring response to therapy is one of the most useful applications of LB in clinical oncology, particularly for those therapies with known resistance mechanisms due to the fact that that anti-*EGFR* resistant clones may be present in the circulation months before progression was clinically obvious. Therefore, the IASLC guidelines suggest the use of LB-first algorithm to detect resistance mechanisms (Rolfo et al., 2018).

1.5. DIGITAL PCR

Since there is a small proportion of ctDNA present in the total cfDNA samples, it is important to select a highly sensitive technique to make therapeutic decisions.

Digital PCR (dPCR) is an end-point PCR method that is used for absolute quantification. Digital PCR has many potential applications, including the detection and quantification of low-level pathogens (Bian et al., 2015) rare genetic sequences (Hudecova, 2015) copy number variations (CNVs) (Day et al., 2013), gene expression in single cells, and quantification of circulating miRNAs expression (Ma et al., 2013).

This process uses similar reagents as the traditional PCR reaction, but the key difference between dPCR and traditional PCR relies on the method of measuring nucleic acids amounts, with the former being a more precise method than traditional PCR (Pohl & Shih, 2004). Aditionally, a key feature of dPCR used in oncology is its ability to detect rare alleles and quantified them in presence of abundant wild-type (WT), without reference of standards or controls and dPCR does not rely on cycle thresholds (Ct), allowing the absolute quantification of molecules and classifying the reactions as positive or negative. Therefore, PCR efficiencies are much less likely to influence the result.

In allelic discrimination assays, the PCR assay includes a specific, fluorescent, dye-labeled probe for each allele. The probes contain different fluorescent reporter dyes (FAM™ and VIC™) to differentiate the amplification of each allele (Figure 7) (Livak *et al.*, 1999).

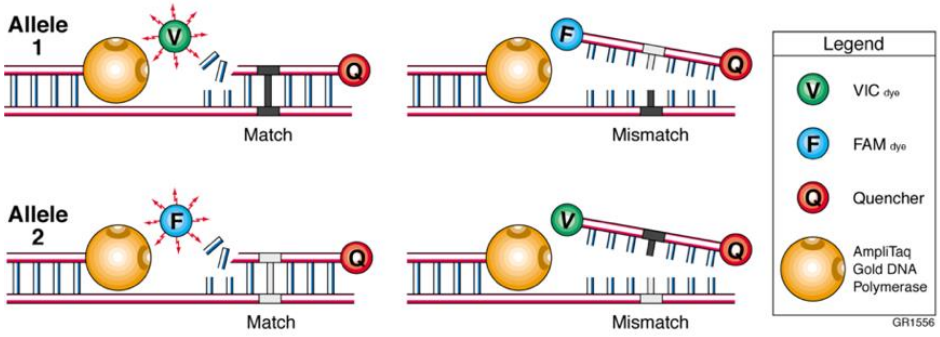


Figure 7. Allelic discrimination assays using TaqMan ® PCR specific probes. In mutant and wild-type detection analysis using dPCR platforms, these probes are used to register different signals from each type of allele. Therefore, an increase of VIC™ fluorescence intensity only indicates the homozygosity of allele 1, whereas the increase of FAM™ fluorescence intensity only indicates the homozygosity of allele 2 and both fluorescent signals indicate the presence of heterozygosity.

However, since cfDNA is composed of multiples sources of DNA, not only tumoral DNA, accurate detection of mutations is very complex. Clonal hematopoiesis is a process that leads to expansion of mutations in peripheral blood cells and, taking into account the high sensitivity of dPCR platforms, false positive plasma genotyping and misdiagnose are probable to occur (Bauml & Levy, 2018).

1.5.1. Sysmex ®Inostics BEAMing Digital PCR System

BEAMing (Beads-Emulsion-Amplification-Magnetics, by *Sysmex Inostics*) is a targeted, quantitative digital PCR technology that employs bead-based amplification in water-in-oil emulsions, and allele-specific hybridization followed by flow cytometry, for the detection of small amounts of mutated DNA released by tumors into the blood circulation. BEAMing is highly sensitive, able to detect mutant ctDNA in very low proportion (as low as 0.01% of total DNA fragments; see Figure 8) in a backgroud of normal (WT) DNA.

First of all, DNA molecules are amplified in a predetermined locus of interest by conventional PCR. Next, amplified DNA molecules are loaded onto magnetic beads coated with specific PCR primers for the gene of interest. A second round of PCR is done on the beads in an oil and water emulsion (emulsion PCR). Once demulsification and magnetic capture are completed, fluorescent-tagged probes specific for either the wild-type sequence or for particular common point mutations are added and hybridize to the amplified DNA. Finally, magnetic flow cytometry is performed to detect the fluorescent tag and quantify the number of beads containing mutated DNA

Since BEAMing is a digital PCR technique that analyzes one allele at a time, it is highly sensitive for the detection of rare mutant alleles within a large population of WT alleles (Lauring & Park, 2011). Nevertheless, BEAMing assay is not full gene or even full exon sequencing and will not identify all mutations in a particular gene (Richardson & Iglehart, 2012). The OncoBEAM ™ *EGFR* Kit of Sysmex® is able to identify 36 cancer relevant *EGFR* mutations present in ctDNA extracted from plasma, including sensitizing mutations such as exon 19 del and L858R, as well as the T790M resistance mutation.

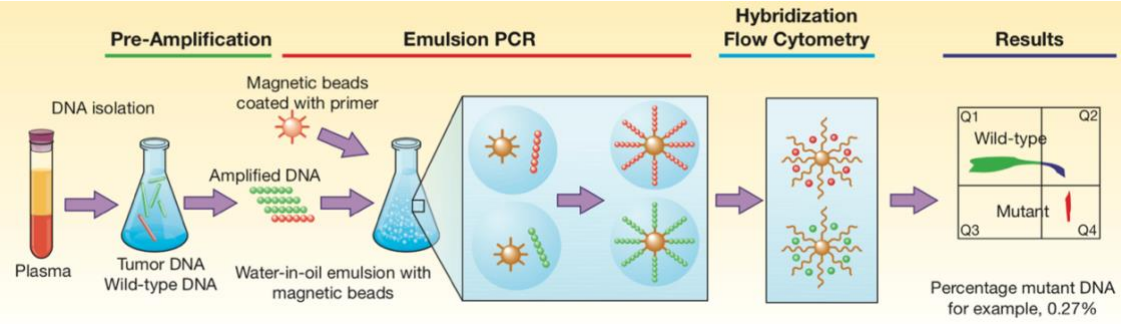


Figure 8. Principles of BEAMing. Shown are the sequential steps involved with BEAMing**.** Results are expressed as a ratio of mutant to wild-type DNA molecules. Flow cytometry provides a quantitative measurement of the mutant DNA present in the plasma (Lauring & Park, 2011).

1.5.2. QuantStudio ™ 3D Digital PCR System

QuantStudio ™ 3D Digital PCR System is a highly sensitive chip-based platform that involves minimal pipetting steps and a sealed system. Therefore, compared to other dPCR platforms such as droplet dPCR, QuantStudio ™ 3D minimizes the level of expertise needed to perform digital PCR, decreasing the likelihood of cross-contamination as well, and the release of amplicons to surfaces and equipment in the lab.

The process (Figure 9) consists on loading the reaction mix onto the uniquely tagged chip, amplification on a dual flat block thermal cycler, and reading of the target concentration in less than a minute on the Applied Biosystems™ QuantStudio™ 3D Digital PCR Instrument.

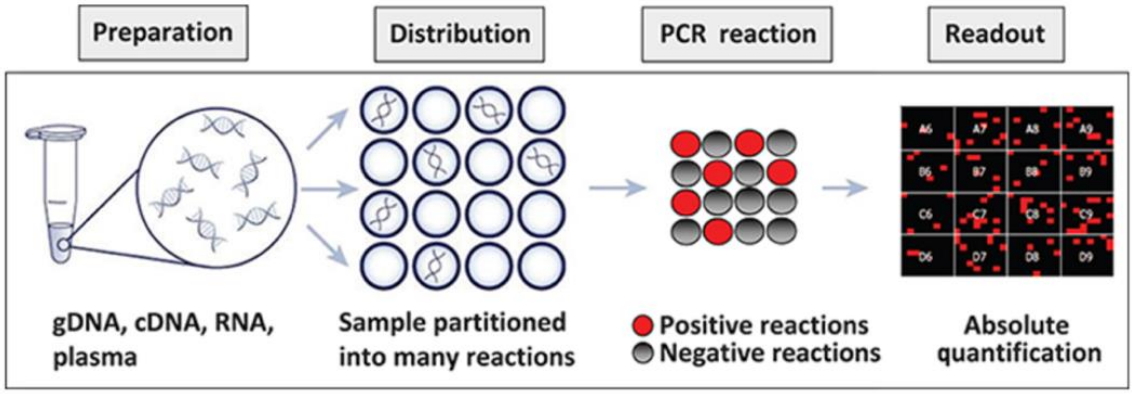


Figure 9. Workflow of QuantStudio ™ 3D Digital PCR System. To perform digital PCR, a nucleic acid mixture is partitioned into many reaction wells, such that some wells receive a target molecule, and some do not. Reactions are subjected to standard PCR to identify wells that have not received target molecules. A standard statistical correction model accounts for wells that may have received more than a single target molecule, and a final concentration value is produced (THERMO FISHER SCIENTIFIC, 2015).

According to 3D digital PCR approach, the reaction is divided into 20,000 individual partitions, and the absolute copy number is calculated based on statistical interpretation of the number of partitions where the target mutation alleles have been detected, compared to those where wild-type alleles have been detected. The results obtained for the samples and amplificated products are easily interpretable (in target copies/ µL).

Moreover, for each answer calculated by the QuantStudio ™ 3D Digital PCR System that determines absolute number (copies/µL), a data quality assessment is made. Data considered to be of marginal or failing quality are then appropriately flagged for further review in secondary analysis.

2. OBJECTIVES

Acquired *EGFR* mutations represent a milestone in lung cancer treatment, especially in advanced NSCLC. Analyses of this mutations in ctDNA have important clinical implications in the management of NSCLC patients in order to have real-time prognostic and predictive information. Therefore, the main objective of this project is to analyze the applicability of liquid biopsy in a cohort of EGFR-mutated advanced NSCLC patients using digital PCR approaches and correlate the analytical parameters with clinico-pathologic and prognostic variables.

To do so, the specific aims of this study are:

1. To assess the capability of different digital PCR platforms to detect epidermal growth factor receptor (*EGFR*) gene mutations, including T790M, L858R and exon 19 deletion, from circulating tumor DNA (ctDNA) in advanced non-small cell lung cancer (NSCLC) patients.

2. To analyze concordance in the *EGFR* mutational status between tissue and liquid biopsies.

3. To monitor the *EGFR* mutational status and allelic fraction in Liquid biopsy (LB) from advanced NSCLC patients during TKI treatment and to correlate these variables with clinical data.

4. To analyze the prognostic and predictive information provided by the *EGFR* status in LB with the patient surveillance.

5. To integrate the results and validate the applicability of liquid biopsy in advanced NSCLC.

3. MATERIALS AND METHODS

3.1. PATIENT COHORT AND BLOOD SAMPLE COLLECTION

Eighteen patients from "Consorcio Hospital General Universitario de Valencia" with *EGFR* mutated adenocarcinoma of the lung, were consecutively included in the present study which was conducted in accordance with the Declaration of Helsinki and the institutional ethical review board approved the protocol. All patients had signed the informed consent prior to the collection of their biological samples.

Patients were treated according to Spanish guidelines with EGFR-TKIs. Plasma samples were collected at the time of diagnosis and throughout standard EGFR-TKIs treatment until progression.

A total of 141 blood samples were collected in EDTA tubes at diagnosis, during follow-up and at progression. Concordance between tissue and plasma *EGFR* mutation status was calculated as the number of positive plasma samples out of the total number of tissue samples. Cases at which T790M was first detected in blood were compared to date of progression as determined by radiological imaging in standard practice.

Moreover, two commercial cell lines of lung cancer (HCC-827H and NCI-H1975) and one cervix cancer cell line (HeLa) as wild-type control, were used for the set-up of dPCR protocols. These cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell line characteristics are illustrated in Table 1.

Table 1. Cell line characteristics.

Cell line	Species	Tissue	Histology	EGFR Mutation
HeLa	*H.sapiens*	Cervix	adenocarcinoma	WT
NCI- H1975	*H.sapiens*	Lung	adenocarcinoma	*EGFR* (L858R, T790M)
HCC-827H	*H.sapiens*	Lung	adenocarcionma	*EGFR* (exon 19 deletion)

3.1.1. Plasma isolation

Peripheral blood samples were collected at the time of diagnosis and throughout standard TKI treatment until progression. A blood volume of 10 mL was collected for each patient in EDTA tubes that were processed for obtaining the plasma fraction. The blood samples were centrifuged at 1600 g during 10 min by means of a tilting trough rotor to separate the different fractions of the blood (Figure 10). Then, the plasma was collected and centrifuged again 10 min at 6000 g to eliminate any blood cells present in the sample. The isolated plasma was stored, at -80 ° C, until further downstream analysis.

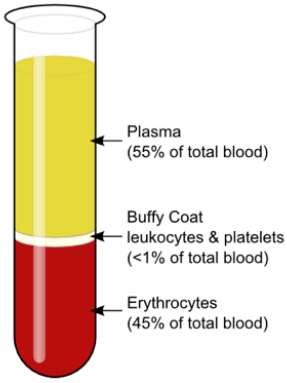


Figure 10. Blood fractions. Blood components can be separated by centrifugation into different fractions depending on their density (from more to less dense components): erythrocytes, buffy coat, and plasma.

3.1.2. DNA extraction

DNA was extracted from 1- 3 mL of plasma using the QIAamp Circulating Nucleic Acid (Qiagen) following the manufacturer instructions.

For the cell lines, they were grown in culture media and their DNA was extracted using the QIAmp DNA Mini and Blood Mini Handbook (Qiagen) for purification of nucleic acids from cultured cells.

Finally, the concentration DNA present in the samples, was quantified using the Invitrogen Qubit 3 Fluorometer device. Additionally, two standard calibrators provided by the manufacturer were also quantified to check the accuracy of the device. The optimal values obtained of the standards used were around 30-50 for the first standard and around 20000 for the second one. The value of these standards and parameters indicated that the protocol is appropriated to obtain good quality DNA for gene expression analysis. The ctDNA was obtained from plasma and *EGFR* mutations were assessed by Sysmex® BEAMing and QuantStudio™ dPCR platforms.

3.2. BEAMING DIGITAL PCR

The mutational analysis of the *EGFR* gene in LB was carried out with the commercial OncoBEAM™ *EGFR* Kit of Sysmex ®, a technique based on digital emulsion PCR that allows the detection of an *EGFR* mutant allele among 10 000 wild ones. Since it is a very sensitive technique, the Molecular Oncology laboratory has two separate rooms for preparing pre-PCR and post-PCR reagents separately to avoid cross-contamination. The main steps of the process included:

1. Preamplification: The preamplification of the *EGFR* regions was performed in a multiplex PCR (mPCR). The DNA sample was diluted 1: 3 for mPCR. After mPCR, six replicates of each sample were diluted 1:20 in another plate.

2. Emulsion PCR (EmPCR): the diluted DNA samples were transferred to the emulsion PCR plate, together with emulsion working reagents (one for each codon). After this step, the EmulsiFIRE solution was added to induce the emulsion, creating millions of PCR compartments (hydrophilic droplets with a single magnetic bead inside) in a single reaction.

3. Hybridization: After the EmPCR, thousands of copies of identical DNA fragments covered each drop. Subsequently, the emulsion was broken to hybridize the template DNA with the specific FAM™ and VIC™ labelled probes (TaqMan®, Applied Biosystems, USA). After hybridization, several washing steps were carried out, to eliminate the unhybridized strands, before analyzing the samples by the Cube6i flow cytometer of Sysmex ®.

4. Flow cytometry: The final step consisted on a flow cytometric analysis of the labelled beads. According to the position of the beads and the fluorescence intensities, the mutated and non-mutated populations could be distinguished. Finally, a set of graphs was generated for the quantification of the number of "beads" mutated in each sample (Figure 11).

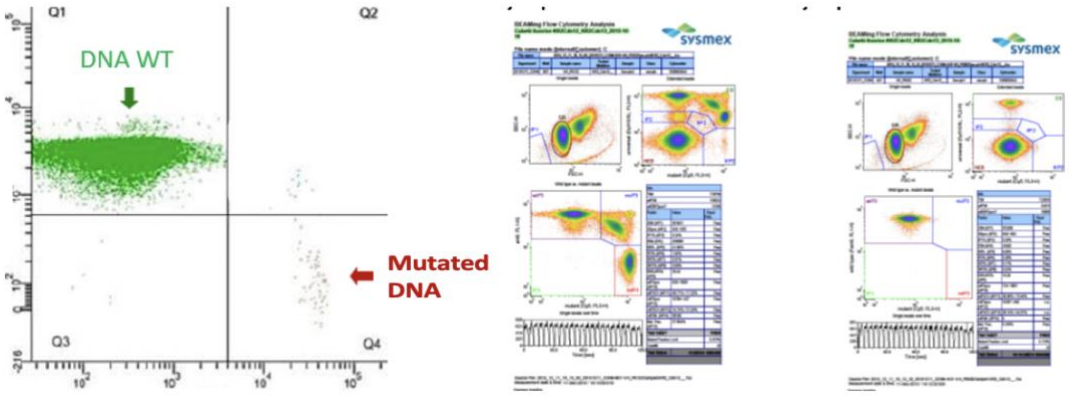


Figure 11. Example of a set of graphs generated in BEAMing digital PCR quantification. In the first graph, the green dots correspond to wild-type DNA, whereas the yellow ones to the mutated DNA. The second and third images are examples of the results obtained for mutated and non-mutated individuals (respectively).

3.3. QUANTSTUDIO ™ DIGITAL PCR

The ctDNA obtained from patients' samples was analysed using digital PCR (dPCR), a cocktail of primers and hydrolysis probes (TaqMan®, Applied Biosystems, USA), designed to hybridized with the *EGFR* mutations L858R, T790M and exon 19 del. These probes were labelled with FAM™ and VIC ™ dyes.

The ctDNA was diluted to obtain a final DNA concentration of 2.3 ng/µL in each chip and the digital PCR reaction was prepared by mixing control DNA, Master Mix provided by the manufacturer, and the corresponding probes using the QuantStudio ™ 3D Digital protocol. Then, 14.8 µL of the PCR reaction were loaded into a QuantStudio ™ 3D Digital PCR 20K Chip, a lid was applied to cover the chip and the assembly was loaded with immersion fluid. Once prepared, the loading port was sealed, and the chip was thermal cycled using the following program (Table 2):

Table 2. PCR thermal cycling protocol for QuantStudio ™ 3D Digital PCR 20K Chip.

PCR Protocol					Cover Temp.	Reaction Volume
Stage 1	Stage 2		Stage 3			
96.0°C	60.0°C	98.0°C	60.0°C	10.0°C	70.0° C	1 nL
0:10:00	0:02:00	0:00:30	0:02:00	∞		
1x (Hold)	39x (Cycles)		1x (Hold)			

Finally, the Digital PCR 20K Chip using the QuantStudio ™ 3D was read using the Digital PCR Instrument and the data was analysed using QuantStudio ™ 3D AnalysisSuite ™ Software. The workflow of the process is summarized in Figure 12.

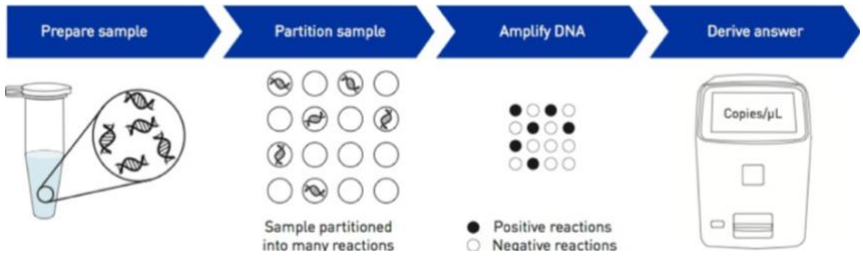


Figure 12. Workflow of QuantStudio ™ 3D Digital PCR (THERMO FISHER SCIENTIFIC, 2015).

Since this technique has not been approved as an in vitro diagnostic (IVD) test yet, the assessment of its limit of detection (LoD) needed. The LoD corresponds to the lowest number of mutant copies that the platform is able to detect. In order to do that, serial dilutions of DNA from *EGFR* mutant and wild-type cancer cell lines were performed.

The DNA extracted from the cell line HCC-827 was used for the analysis of the exon 19 deletion in *EGFR*. Serial dilutions mixing DNA from HCC-827 with wild-type DNA obtained from HeLa cell line, were performed in the following concentrations: 1%, 0.5%, 0.1% and 0.05% (or 0.01%) of mutant DNA. Then, for mutations L858R and T790M, the same procedure was followed but with the cell line NCI-H1975 which contains both mutations and the corresponding probe in each case.

All DNA extracted was quantified using the Invitrogen Qubit 3 Fluorometer and diluted to concentrations approximately of 15 ng/ µL.

3.4. DATA ANALYSIS

Prior to statistical analyses, expression data were carefully reviewed, and those values considered as outliers were excluded. The patient cohort description was studied to obtain the frequencies within the studied population.

Concordance was determined by comparing plasma *EGFR* mutation results to those from matched tumor samples. Statistical analyses between *EGFR* mutational status and clinicopathological variables were conducted by nonparametric tests. Moreover, survival analysis was performed using the Kaplan - Meier method, and the statistical significance between the survival curves was evaluated by the log-rank test. In all cases, basal date was considered at the time of *EGFR* mutation was first detected in advanced patients. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 15.0 software (Chicago, IL), considering statistically significant a p-value < 0.05.

4. RESULTS AND DISCUSION

4.1. COHORT OF ADVANCED NSCLC PATIENTS. DESCRIPTIVE

This study comprised 18 patients with NSCLC in advanced stages (IIIB-IV) at the time of inclusion in the cohort, who were treated and monitored at "Consorcio Hospital General Universitario de Valencia". All of them were *EGFR* mutated and were treated with EGFR-TKIs. The median age of patients was 70 years, 72.2 % were females and 38.9 % were current or former smokers. The treatments for each patient were determined according to the molecular diagnosis of the tissue biopsies, being gefitinib the TKI most frequently used, in 12 (66.6%) cases. The most relevant demographic and clinico-pathological characteristics of the cohort are shown in Table 3.

Table 3. Clinico-pathological features of the cohort.

Clinico-pathological features		Population N=18
Age diagnosis	Median [range]	70 [47-85]
Gender	Male	5 (27.8%)
	Female	13 (72.2%)
Smoking status	Never smoker	11 (61.1%)
	Former smoker	4 (22.2%)
	Active smoker	3 (16.7%)
Exitus	No	13 (72.2%)
	Yes	5 (27.8%)
Tumour Histology	ADC	16 (88.9%)
	Unknown	2 (11.1%)
EGFR mutational status	exon 19 del	7 (38.9%)
	L858R	11 (61.1%)
Stage at diagnosis	I	2 (11.1%)
	II A	1 (5.6%)
	III B	2 (11.1%)
	IV	13 (72.2%)
First line treatment	Afatinib	1 (5.6%)
	Erlotinib	5 (27.8%)
	Gefitinib	12 (66.6%)
Progression	Yes	11 (61.1%)
	No	7 (38.9%)
Second line treatment	Osimertinib	7 (38.9%)
	Others	2 (11.1%)
	Chemotherapy	1 (5.6%)
	None	8 (44.4%)

4.2. DIGITAL PCR PLATFORMS COMPARISON

Nowadays, there are several PCR based diagnostic platforms that are available for *EGFR* mutation detection including cobas ®, ARMS, BEAMing and NGS among others (Li & Zhou, 2017). Nevertheless, not all of them have the same sensitivity when analyzing liquid biopsies of patients with relatively low mutant allelic fraction. In this context, dPCR platforms outperformed in sensitivity and specificity the non-digital ones (Thress et al., 2015). Therefore, one of the aims of our study was to compare two different dPCR platforms for *EGFR* mutation detection in liquid biopsies, BEAMing and QuantStudio ™.

First of all, determination of the optimum threshold or LoD should be considered for best development of diagnostic tests. As explained before, the OncoBEAM ™ *EGFR* Kit of Sysmex® is able to identify 36 cancer relevant *EGFR* mutations present in ctDNA extracted from plasma using BEAMing technology. This kit was launched by Sysmex ® at the end of 2018, providing an analytical performance with high sensitivity. The LoD of the mutant fraction in this platform for all sensitizing mutations is 0.03 %, whereas for all resistance ones is 0.04 %.

On the other hand, since QuantStudio™ 3D dPCR has not been yet approved as an IVD test, several quantifications were performed to obtain the LoD of this platform. This technique has a promising future since it has a low detection limit, is easy to carry out and is cheaper than other technologies used in the laboratory nowadays (Conte et al., 2015)

4.2.1. Serial dilutions to calculate the LoD of QuantStudio ™ 3D dPCR System.

To validate the LoD of the QuantStudio ™ 3D Digital PCR System for the *EGFR* T790M mutation, the ctDNA extracted from the cell line NCI-H1975 was used. Serial dilutions mixing ctDNA from NCI-H1975 with wild-type cfDNA obtained from HeLa cell line, were performed in the following concentrations: 1%, 0.5%, 0.1%, 0.05% and 0.01% of mutant cfDNA (Figure 13). Moreover, the cell line NCI-H1975 was also used to detect the LoD for the *EGFR* L858R mutation. Serial dilutions mixing ctDNA from HCC-827 with wild-type cfDNA obtained from HeLa cell line were performed in the following concentrations: 1%, 0.5%, 0.1% and 0.01% of mutant cfDNA.

On the other hand, to validate the LoD for the *EGFR* exon 19 del mutation, the ctDNA extracted from the cell line HCC-827 was used. Serial dilutions mixing ctDNA from HCC-827 with wild-type cfDNA obtained from HeLa cell line were performed in the following concentrations: 1%, 0.5%, 0.1% and 0.01% of mutant cfDNA.

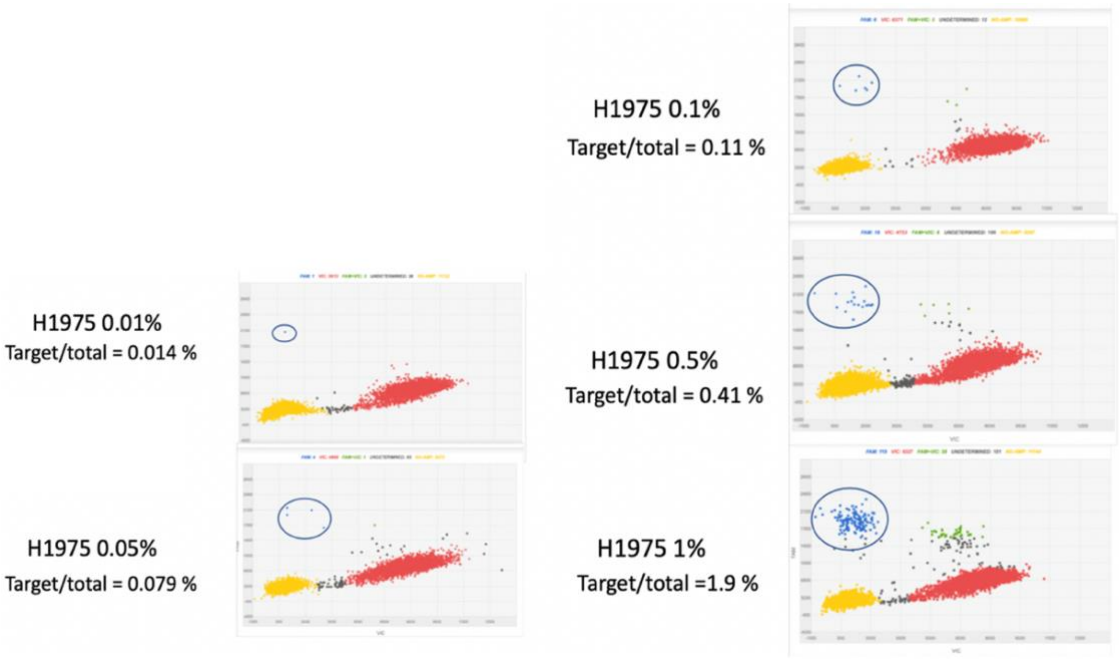


Figure 13. Example of serial dilutions to calculate the LoD for the T790M *EGFR* mutation. The blue dots represent the mutant alleles that have hybridized with the FAM™ probe, whereas the red ones indicate the wild-type alleles that have hybridized with the VIC™ probe. The yellow dots are non-amplificated regions and the green ones indicate amplicons that have both copies (WT and mutant). Finally, the grey dots indicate undetermined spots that do not pertain exactly to any region of the plot.

The LoDs and the thresholds stablished for each of the *EGFR* mutations are shown in Table 4.

Table 4. Calculated threshold for FAM™ and VIC™ probes signal intensity and Limit of Detection for each *EGFR* mutation.

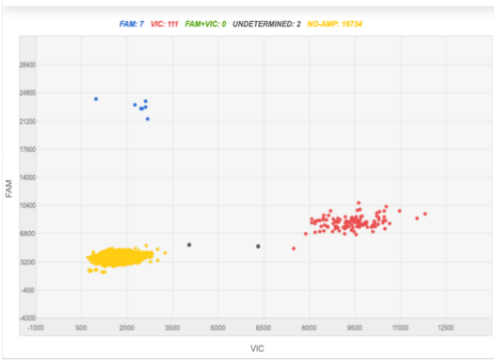
Mutation	FAM™ treshold	VIC™ treshold	LoD
T790M	15741.8	3529.15	0.026%
L858R	3456.01	3294.39	0.0132%
exon 19 del	9837.61	3510.29	0.00716%

4.2.2. Patient monitoring using BEAMing and QuantStudio™ dPCR Systems. Comparative

In order to compare the performance of BEAMing and QuantStudio™, blood samples from different patients of the cohort were analyzed using both platforms. The mutant fraction obtained in each of them differs as a consequence of the calculation parameters. On one hand, the mutant allelic fraction (MAF) determined in BEAMing is calculated by counting mutant versus WT beads and their ratio or proportion. On the other hand, the target/total obtained in QuantStudio™ is calculated as the proportion between the target molecules that have bound with the specific FAM™ probe and the total number of molecules (WT + mutants) that were in the reaction. The comparison of samples from 2 patients in both platforms are described in more detail.

A fraction of the plasma sample from patient 1 (Figure 14) was analysed using a specific probe for the T790M resistance mutation and the mutant fraction obtained in the sample was 5.915% in QuantStudio™ whereas a determination of the same sample using BEAMing obtained a mutant allelic fraction of 8.31%.

Target/total = 5.915 %



BEAMing MAF: 8.31 %

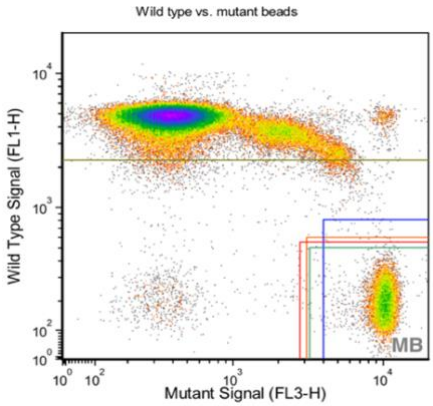
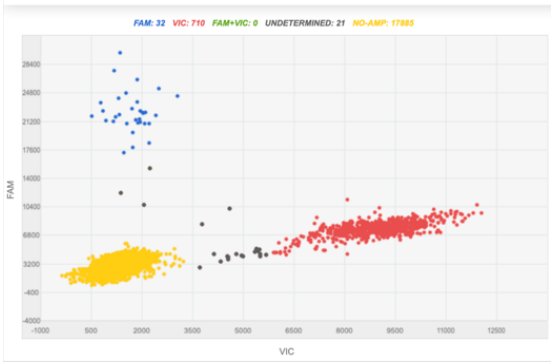


Figure 14. QuantStudio ™ and BEAMing dPCR results for patient 1.

The result presented in Figure 24 indicates a genetic resistance to EGFR-TKIs that the patient developed and that was the cause of the relapse and the consequent change of the first line treatment with gefitinib to a second line treatment using osimertinib. Although the results are slightly different, both of them indicate a considerable presence of the acquired resistance mutation T790M in the plasma fraction of the patient, and a treatment change to a 3rd generation EGFR-TKI should be considered.

In Figure 15, patient 2 experimented a relapse after the first line treatment and some analytical tests were performed to determine a new clinical strategy. The probe selected was specific for T790M, which is the common resistance mutation found in patients who develop resistance to EGFR-TKIs (Yun et al., 2008). The determination for QuantStudio™ platform was 4.246%, whereas for BEAMing was 7.33%. The comparison of the values is again slightly different but both platforms indicates the presence of the T790M in a considerable amount.

Target/total = 4.246%



BEAMing MAF= 7.33%

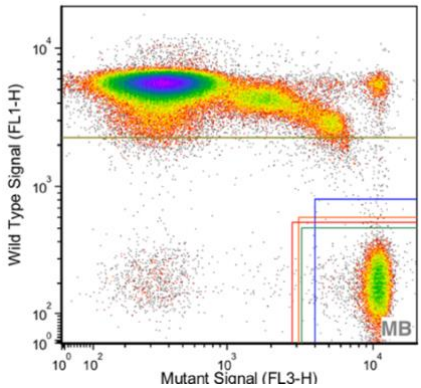


Figure 15. QuantStudio ™ and BEAMing dPCR results for patient 2.

4.3. CONCORDANCE ANALYSIS BETWEEN TISSUE AND LIQUID BIOPSIES

4.3.1. *EGFR* mutational status in tumor biopsy

The analysis of the *EGFR* mutational status of the tissue samples was performed using a sensitive qPCR method based in the Amplification Refractory Mutation System (ARMS), an allele-specific amplification technology approved for clinical diagnosis. From the 19 samples, all of them were *EGFR* mutated, being 11 (61.1%) of them L858R mutated and 7 (38.9%) exon 19 del mutated. These two mutations account for more than 85% of clinically important *EGFR* mutations associated with responsiveness to TKIs (Yung et al., 2009, Novello et al., 2016). Therefore, treatment strategy was made considering these results.

Moreover, 4 patients that progressed after TKIs treatments were re-biopsied, in one case, the L858R mutation was found but the acquired T790M mutation was not detected, whereas in the other 3 cases, the T790M mutation was found (one in concomitance with the sensitizing L858R mutations, and the others two with exon 19 del).

4.3.2. *EGFR* mutational status in liquid biopsy

For this study, the mutant allelic fraction (MAF) was analyzed for each of the patients using BEAMing dPCR technology, because its clinical and analytical performance has been more studied than QuantStudio™.

The cfDNA was extracted from plasma fraction of blood samples obtaining an optimal DNA concentration for all the samples, the median was 0.308 ng/µl [0.156 – 0.658]. From the 18 samples at a first determination, 7 (38.9 %) samples had not detected mutations, 8 (44.4%) had L858R mutations and 3 (16.7 %) harbored exon 19 del mutation. Several determinations using this assay have been done in the sample cohort in order to monitor the *EGFR* mutational status of the tumor during treatment (Figure 16), with special emphasis on the presence of mutations involved in acquired resistance to EGFR-TKIs.

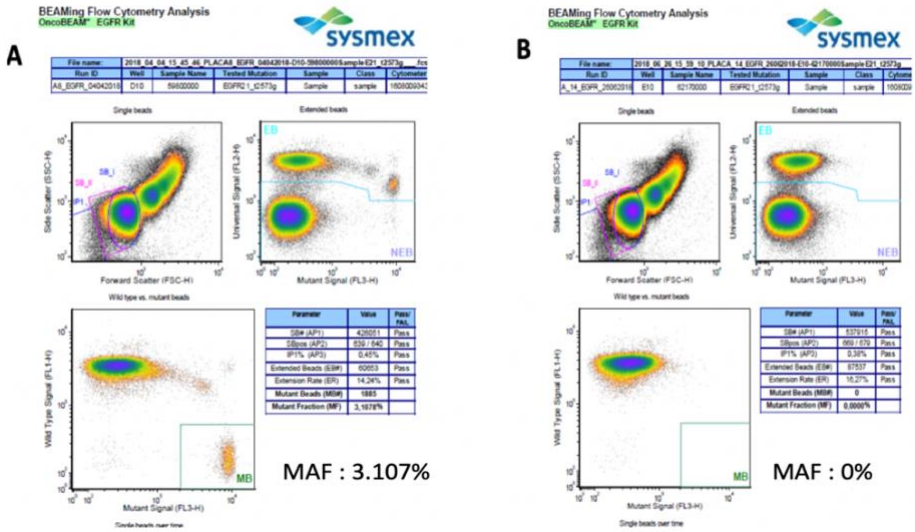


Figure 16. Example of BEAMing determination results for a patient mutated in L858R. In (A), the mutant allelic fraction (MAF) of L858R observed was 3.107%, whereas in another determination (B), there is no a significant number of mutant molecules, giving a wild-type (WT) result.

4.3.3. Concordance analysis between tissue and liquid biopsy

In total, eighteen EGFR-mutated patients of the cohort were compared using ARMS assay for tissue biopsy and BEAMing technology for liquid biopsy (Table 5) at the time of diagnosis.

Table 5. Results of *EGFR* mutations determined by tissue and liquid biopsy and their concordance. In green color are represented the results that are equal for both assays, whereas in red are represented the contradictory results.

Patient	Tissue biopsy	Liquid biopsy
1	L858R	L858R
2	L858R	L858R
3	exon 19 del	exon 19 del
4	L858R	L858R
5	L858R	No mutation detected
6	L858R	L858R
7	exon 19 del	exon 19 del
8	L858R	No mutation detected
9	exon 19 del	No mutation detected
10	L858R	L858R
11	exon 19 del	No mutation detected
12	L858R	L858R
13	L858R	L858R
14	L858R	No mutation detected
15	exon 19 del	No mutation detected
16	L858R	L858R
17	exon 19 del	No mutation detected
18	exon 19 del	exon 19 del

The concordance between blood/tissue samples was 61.1%, with 7 contradictory results in patients 5, 8, 9, 11, 14, 15 and 17, who were *EGFR* mutant positive in the tissue biopsy, but the mutation was not detected in cfDNA.

Those mismatches could be explained by the low concentration of ctDNA present in the plasma samples from these patients, being patient 5 the one with the lowest ctDNA concentration within this subgroup of patients (0.227 ng/ µL). In this context, there are several factors that may influence the release of ctDNA in NSCLC patients.

Firstly, the release of ctDNA to systemic circulation has been correlated with tumour volume, being higher when tumors are bigger (Abbosh et al., 2017). Moreover, it has been discovered that subclones of cancer cells carrying different mutations release ctDNA in distinctive ways, adding a new dimension to tumor heterogeneity (Mao et al., 2017).

Another cause that may be related to these disparities is the fact that some of the patients had residual micro-metastatic disease, or minimal residual disease that have not been eradicated by adjuvant systemic therapy and surgery. Therefore, there is lack of proliferation and apoptosis of the cancer stem cells, causing almost any release of ctDNA (Garcia-Murillas et al., 2015).

Finally, cancer patients with brain metastases, have been also correlated with a lower amount of ctDNA molecules in their circulatory system. This could be explained because physical obstacles such as the blood-brain barrier and mucin could prevent ctDNA from entering the circulation (Bettegowda et al., 2014, De Mattos-Arruda et al., 2015).

Although the value of the concordance between blood/tissue biopsies obtained in this study is not very significant (61.1%), several articles in this line have obtained relatively high concordance. One example is the lungBEAM Trial led by Pilar Garrido in 2017. This study recruited 109 patients with advanced NSCLC with *EGFR* mutations in order to study the concordance between tissue and plasma baseline and the detection of resistance mutations during the monitoring. Their initial results showed a high concordance at baseline of 71 %.

Another study conducted by Yung et al., in 2009 had similar concordance results between the tissue and liquid biopsies. *EGFR* mutations were detected in the tumor tissues of patients using conformation-sensitive gel electrophoresis (CSGE) and sequencing analysis. Corresponding mutations were detected in the plasma using chip-based dPCR and obtaining a concordance of 92%.

Regarding the use of ARMS technology for *EGFR* mutation detection, Liu et al., in 2013 and Douillard et al., in 2014 tested also the concordance rate between tissue and plasma biopsies obtaining 84.9 % and 94.3 %, respectively.

4.4. PATIENT MONITORING USING BEAMING TECHNOLOGY

In this study, monitoring through the analysis of ctDNA in 18 patients was performed. During the disease course, a total of 141 liquid biopsies were collected at different time points in the disease course. Temporal changes of *EGFR* mutation levels in plasma DNA from advanced NSCLC patients are schematically shown in Supplementary Table 1.

The samples were analysed using BEAMing technology and focusing on two transitions: transition due to EGFR-TKI treatment initiation and, if the case, after acquiring EGFR- TKI resistance.

Patients 1 to 17 were treated initially with a first-generation EFGR-TKI (erlotinib or gefitinib), whereas patient 18 was treated with a second-generation EFGR-TKI (afatinib). All these treatment strategies were followed according to the European Society for Medical Oncology (ESMO) Clinical Practice Guidelines for diagnosis, treatment and follow-up of patients with advanced NSCLC (Novello et al., 2016).

Some of the patients of the cohort presented tumour metastases together with other complications such as thoracic adenopathy or pleural effusion. The more common sites of metastatic disease in NSCLC include liver, bones, adrenal, brain, and contralateral lung (Socinski & Morris, 2003). In this case, bone metastases were detected in 7 patients (patients 1, 2, 4, 10, 15, 17 and 18). Additionally, 3 patients (patients 1, 2 and 12) had brain metastases detected after the surgical resection. The first-generation EGFR-TKIs, gefitinib and erlotinib, are interesting options in bone and brain metastases but only a few studies have been conducted. Gefitinib seems to have important effects against bone resorption as well as antitumor effects (Antonio et al., 2014). Therefore, a total of 12 patients (66.7%) were treated with gefitinib, which is as well one of the treatments recommended by the Spanish Society of Medical Oncology (SEOM) clinical guidelines for the treatment of *EGFR*-mutated NSCLC (Corral & Vin, 2015).

4.4.1. *EGFR* mutational status assessment.

Regarding *EGFR* mutations detected in the patient cohort, the sensitizing mutation L858R was found in 11 patients (patients 1, 2, 4, 5, 6, 8, 10, 12, 13, 14 and 16), whereas the exon 19 deletion was present in the other 7 patients (patients 3, 7, 9, 11, 15, 17 and 18). The mutant allelic fractions (MAF) detected varied depending on the subtype of *EGFR* mutation and the disease course.

For patients L858R mutated (Figure 17), BEAMing results for the MAF were plotted against time. At the time before treatment, the average MAF was 1.24% [0.11– 5.23] and the average ctDNA concentration was 0.343 ng/µL [0.194 – 0.912].

From the 11 patients, only patients 1, 2, 5, 8 and 14 experimented a considerable decrease of their mutant fraction in an average of 85 days. All of them were treated with the EGFR-TKI gefitinib as first line. Nevertheless, although gefitinib is known as an efficient drug to treat advanced NSCLC (Jackman et al., 2006), it can also present some toxicity to patients (Wo et al., 2018), and the treatment should be changed before it finishes all the cycles agreed. This was the case of patient 8, who had a change from gefitinib to erlotinib and experimented a good response.

In the case of patient 13, she had a reduction of their mutational charge taking erlotinib as her first line EGFR-TKI but, after approximately three months, she had another increase of the L858R mutational charge accompanied by the acquisition of the T790M resistance mutation. Finally, patients 4, 6, 10, 12 and 16 had an unsuccessful treatment since their mutational charge increased, causing the death of patient 6 and the relapse of the other four patients.

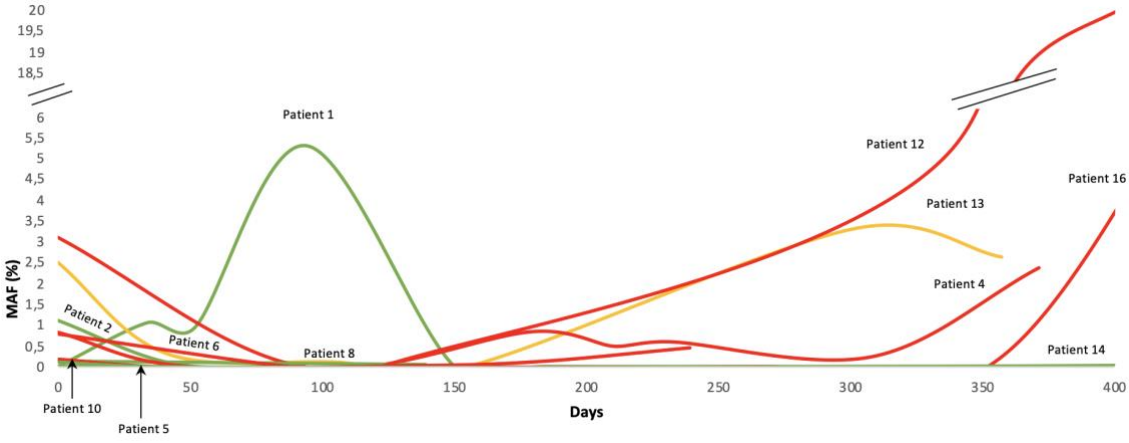


Figure 17. L858R mutated-patient monitoring using BEAMing technology. The change in the MAF (in %) of the patients is represented during time (in days). Patients who had a good response to treatment are in green, patients who had a good response but the MAF increased again are in yellow and, patients who had a negative response are in red.

For patients mutated with an exon 19 del (Figure 18), BEAMing results for the MAF were plotted against time. The average MAF at pretreatment was 5.53% [0.021 – 20.01] and the average ctDNA concentration 0.326 ng/µL [0.144 – 0.844].

In this case, patients 3, 7, 15, 17 and 18 experimented a good response to the administered EGFR-TKI in an average of 111 days. Patients 3 and 7 had gefitinib, whereas patients 15 and 17 had erlotinib and patient 18, afatinib. Afatinib was chose since it has showed a significant

improvement of the overall survival of patients with exon 19 del than those that had only chemotherapy (Byrne, 2015). In this example, the drug acted efficiently since the mutational charge diminished considerably.

On the other hand, patients 9 and 11, who were treated with gefitinib, had an increment of their mutational charge which ended up in deterioration of patient 9 and death of patient 11.

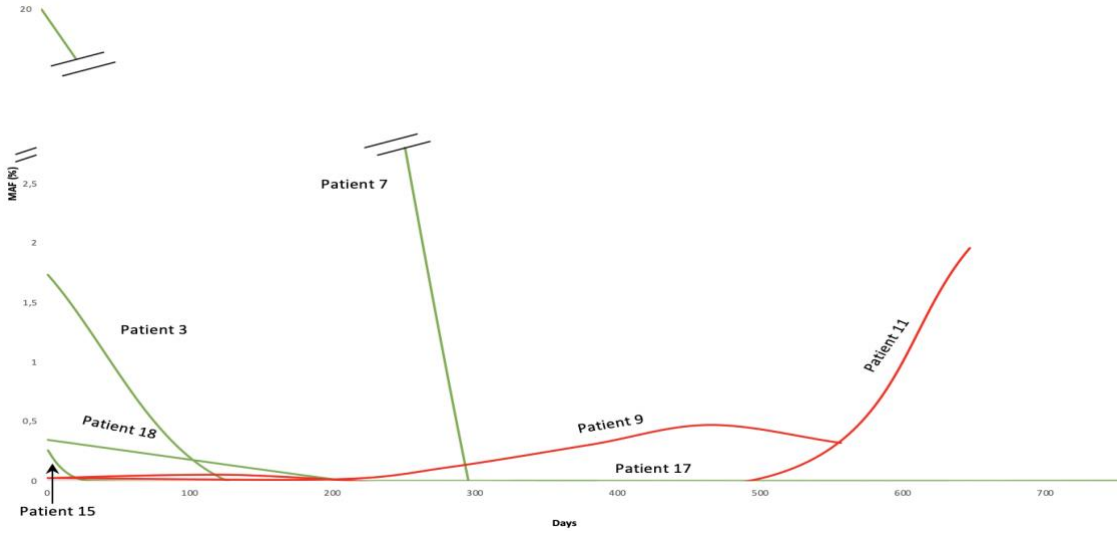


Figure 18. Exon 19 deletion mutated-patient monitoring using BEAMing technology. The change in the MAF (in %) of the patients is represented during time (in days). Patients who had a good response to treatment are in green, patients who had a good response but the MAF increased again are in yellow and, patients who had a negative response are in red.

4.4.2. Sensitizing and T790M resistance mutations tracking to monitor disease progression

Despite a striking initial response to treatment, with a considerable reduction of the mutational charge, practically all NSCLC patients experience disease progression generally after 10–14 months of treatment (Planchard et al., 2015). The *EGFR* mutation T790M is the most common secondary mutation responsible of acquisition of resistance to EGFR-TKIs that occurs in exon 20 of the *EGFR* gene (Morgillo et al., 2016, Yi et al., 2017). Besides, some studies have shown that in patients with tumors bearing gefitinib- or erlotinib-sensitive *EGFR* mutations, resistant subclones containing an additional *EGFR* mutation (T790M) emerge in the presence of these drugs (Pao, William; Chmielecki, 2010).

During the study follow up, 10 patients (patients 1, 6, 10, 11, 12, 13, 15, 16, 17 and 18) had a disease progression that was detected by CT-scanning (Figure 19). Moreover, 8 of them (80%) had the presence of the T790M resistance mutation in the liquid biopsy determinations, remarking that the majority of these tumors presented again the sensitizing mutation. In these 8 patients' plasma, T790M-positivity was detected in an average of 14 days prior to radiological progression, whereas the first sensitizing mutation detection was in an average of 67 days prior to radiological progression.

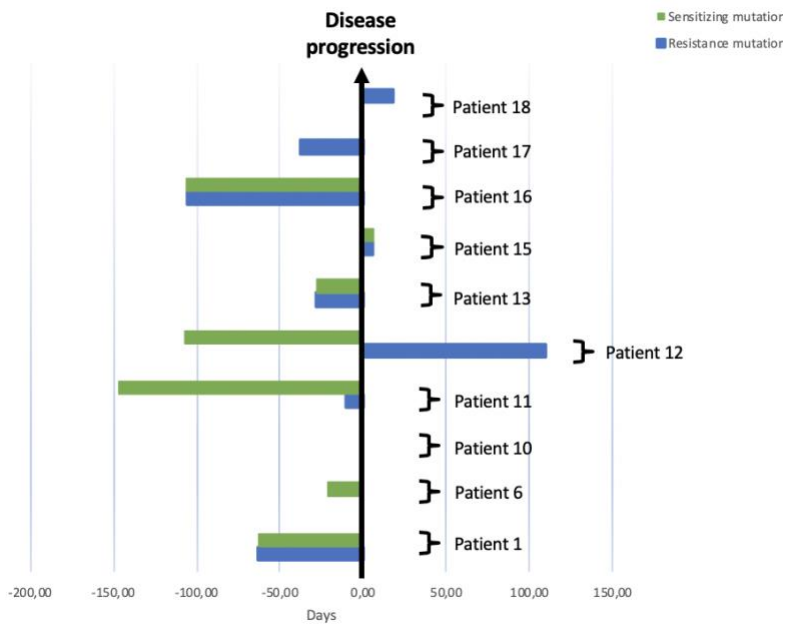


Figure 19. Number of days since the earliest identification of the T790M mutation and earliest identification of an increment of sensitizing mutation in the blood and assessment of disease progression by CT-scan. The blue bars represent the T790M resistance mutation, whereas the green bars, the sensitizing mutation (L858R or exon 19 del).

This T790M acquired resistance has been related to the use of EGFR-TKIs (Clark et al., 2005, Planchard et al., 2015). In particular, monitoring T790M is important to predict the presence of acquired resistance. A new generation of EGFR-TKIs, designed for T790M-positive *EGFR*, requires the selection of patients on the basis of their T790M status (Uchida et al., 2015), which is a third-generation EGFR-TKI specific aimed to patients with T790M-positive advanced NSCLC in whom disease had progressed during first-line EGFR-TKI therapy (Mok et al., 2016). Therefore, the patients that showed this mutation were more prone to have a relapse, and the second line treatment chosen for most of them was the third-generation drug osimertinib.

Provencio et al. in 2017 performed a similar analysis tracking T790M mutation to monitor treatment outcome. On average, they detected this mutation in plasma 51 days before the assessment of progression disease by CT-scan and concluded that the detection of the T790M mutation in blood, together with an increase of the original sensitizing *EGFR* mutation in serial plasma samples, was significantly associated with progression disease diagnosis.

Nevertheless, the T790M mutation seems to be underrepresented in the tumor cell genome relative to the total number of *EGFR* alleles, and it is detectable in approximately 50% of patients with NSCLC who relapse after an initial response to TKIs. This suggests that T790M might either be present in only a subset of resistant cancer cells, or might be present only in a minority of copies of the *EGFR* gene in each tumor cell (Inukai et al., 2006, Sharma et al., 2007).

On the other hand, the comparison of MAFs before the treatment and at the time of progression showed similar values for sensitizing mutations, being the average MAF for sensitizing mutations at pretreatment of 0.78%, whereas the same measurement at the time of progression was 0.79%. Nevertheless, the mutant fraction of T790M is detected at lower values, being the average MAF 0.31% (Figure 20).

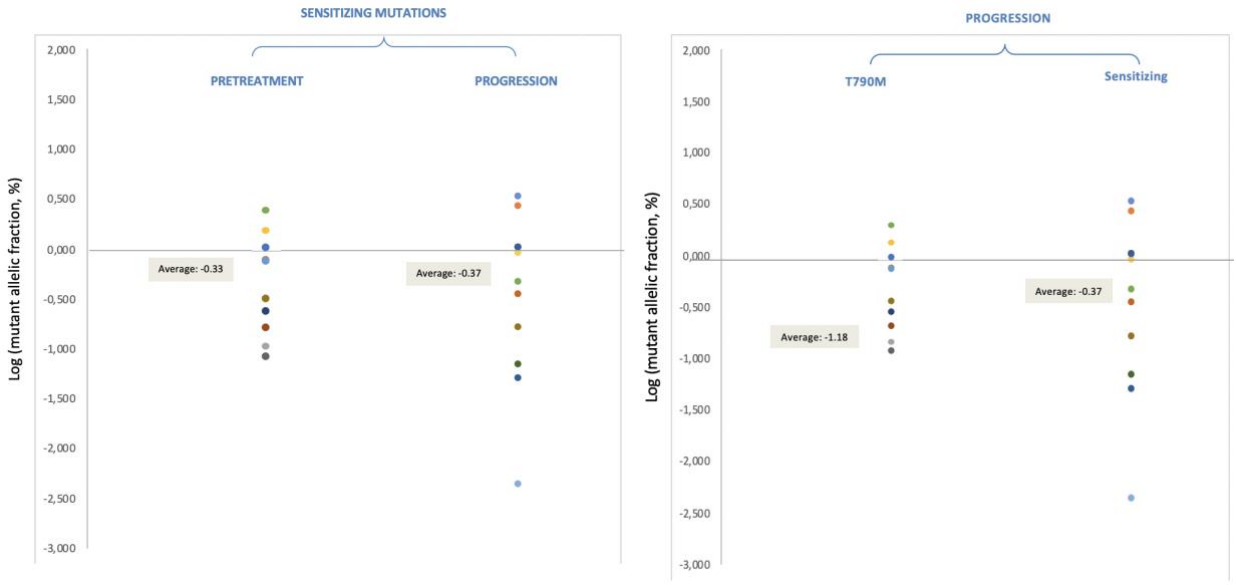


Figure 20. Mutant allelic fraction (MAF) of NSCLC patients with progression disease detected before the treatment (sensitizing mutation) and at the time of progression (sensitizing and T790M mutations). In the both plots, the MAF is represented as the logarithmic percentage and its corresponding average in logarithmic value for each situation. The plot at the left side represents the MAF of the sensitizing mutation (L858R or exon 19 del) pretreatment and at the time of progression of the 10 patients. The other plot represents the MAF at the time of progression for the sensitizing and resistance mutations (in 8 patients that presented the T790M mutation).

4.4.3. Use of NGS to complement patient monitoring with BEAMing technology

Since mutation evaluation in NSCLC includes a wide spectrum of mutated genes apart from *EGFR*, several sophisticated techniques such as NGS evaluation should be used to find new mutations in progressed patients. For maximal sensitivity and specificity in a wide panel of genes, the Oncomine Pancancer Assay for LB in NGS was used in samples from 2 patients of the cohort that had a relapse without showing an apparent increase in the T790M mutation.

Firstly, patient 13, she was 85 years old women who had never smoked. At the time of diagnosis, the tumour was and ADC at stage IIIB unresectable with the presence of the L858R mutation (Figure 21). The treatment of choice for this patient was erlotinib, which was one of the EGFR-TKIs recommended by the Spanish Society of Medical Oncology (SEOM) clinical guidelines for the treatment of NSCLC (Corral & Vin, 2015) in *EGFR*-mutated patients.

This patient did not have metastases after surgical resection, and the mutational charge diminished considerably. After 11 months of treatment with erlotinib, the patient suffered unfortunately a pericardial effusion which was related to an increase in both L858R and T790M mutations. Malignant pericarditis, when associated with massive pericardial effusion, presents a critical condition in lung cancer patients. Therefore, different approaches should be done to treat it, which include percutaneous pericardiocentesis combined with the draining of effused pericardial fluid and topically administering carboplatin (Moriya et al., 2000).

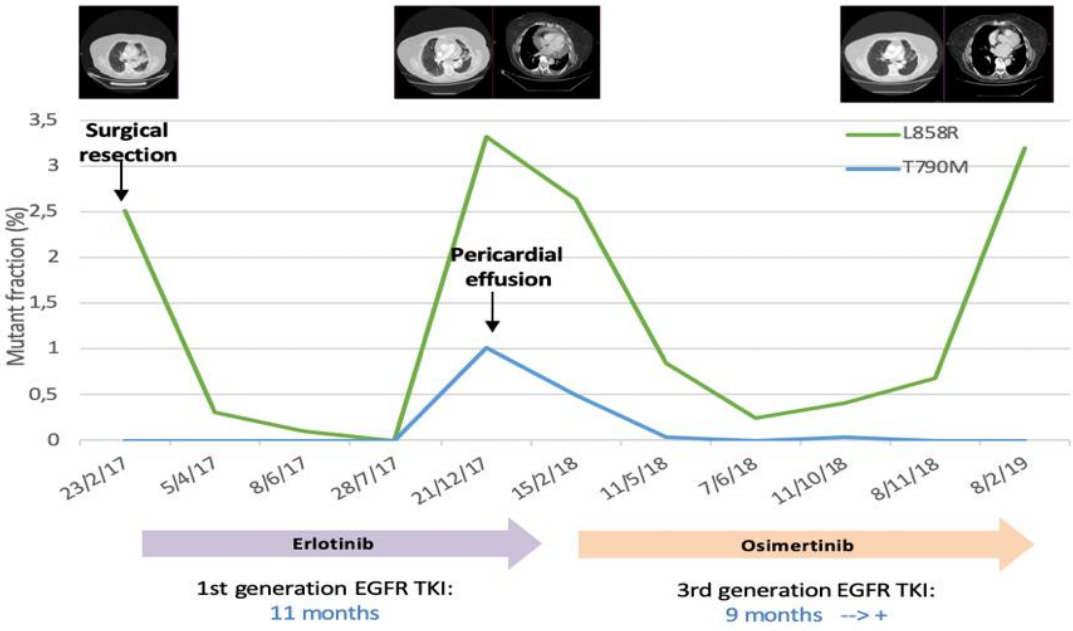


Figure 21. Monitoring of patient 13 related with the mutant fraction (%) present in liquid biopsy samples.

This patient did not have metastases after surgical resection, and the mutational charge diminished considerably. After 11 months of treatment with erlotinib, the patient suffered a pericardial effusion which was related to an increase in both L858R and T790M mutations, which presented a MAF of 3.32% and 1.01%, respectively. Malignant pericarditis, when associated with massive pericardial effusion, presents a critical condition in lung cancer patients. Therefore, different approaches should be done to treat it, which include percutaneous pericardiocentesis combined with the draining of effused pericardial fluid and topically administering carboplatin (Moriya et al., 2000).

In this context, it can be suggested that an increase of activation of T790M mutation may correlate with disease progression and response to EGFR-TKIs, as other studies have corroborated in the last years (Oxnard et al., 2016, Oya et al., 2017).

As a consequence, the treatment was changed and the second line drug chosen was osimertinib, which is the recommended drug for patients that have had a relapse after previous treatment with an EGFR-TKI and have confirmed T790M. The therapeutic strategy generated a good response in the patient, with a considerable reduction of the MAF of both mutations, achieving a disappearance of the T790M mutation. Nevertheless, after approximately 5 months, the patient had another increase in the sensitizing mutation L858R.

Since the T790M mutation clones were not present in a significant number, new mutations had to be analysed. Therefore, NGS using the Oncomine Pancancer Assay was performed and a new mutated gene was discovered in this patient. The mutated gene was *BRAF* (MAF 0.32%) which is a gene commonly present in NSCLC patients who are resistant to EGFR-TKIs. Moreover, *BRAF* mutations have been associated with poor prognosis (Marchetti et al., 2011, Zheng et al., 2015)

On the other hand, patient 15 was 76 years old female who had never smoker and she had a poor differentiated lung adenocarcinoma at stage IV, indicating the severity of the disease and the appearance of tumour dissemination (Figure 22). In this case, the patient suffered from bone and hepatic metastases.

Although gefitinib is known as an efficient drug to treat advanced NSCLC, it can also present some toxicity to patients (Wo et al., 2018), and the treatment should be changed before it finishes all the cycles agreed. This was the case of patient 15, who had a change from gefitinib to erlotinib and experimented a good response until approximately 12 months, when it has a relapse and the acquisition of T790M resistance mutation. For that reason, the treatment was again changed to the 3rd generation EGFR-TKI osimertinib, although there is no clinical evidence of a better outcome for those patients with metastases using this drug (Neal et al., 2017).

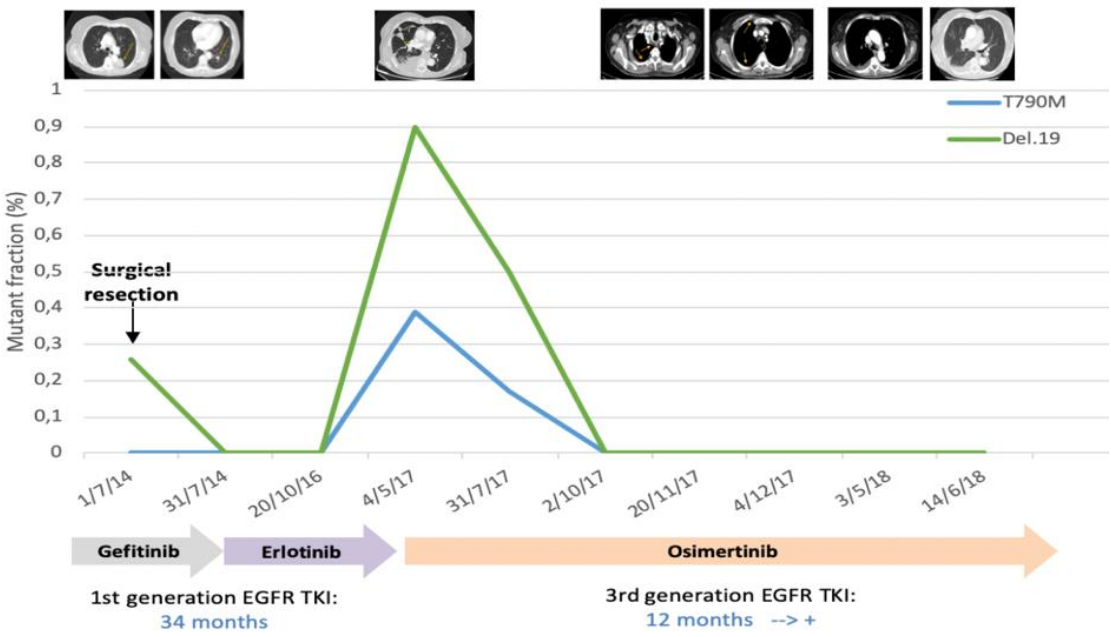


Figure 22. Monitoring of patient 15 related with the mutant fraction (%) present in liquid biopsy samples.

Patient 15 experimented also a relapse when treating with osimertinib which was not related to T790M resistance mutation. In this case, the same NGS Oncomine Pancancer Assay was used, obtaining a new mutation in *TP53* gene with a MAF of 0.2%.

The tumor suppressor gene *TP53* is frequently mutated in human cancers and it is among the most frequent and important events of the various genetic alterations in lung cancer (Mogi & Kuwano, 2011) and it has been related with NSCLC patients that presented progression disease (Scoccianti et al., 2012). Nevertheless, further studies with larger cohorts should be done to determine if comprehensive molecular profiling of the *TP53* gene adds clinically relevant information to single gene assay identification in oncogene-driven lung cancers (VanderLaan et al., 2017).

4.4.4. Survival analysis according to *EGFR* mutational status

To examine the prognostic role of *EGFR* as an independent factor, a survival analysis following the Kaplan – Meyer method was performed. The sample cohort was divided into two groups according to the *EGF*R mutation that they had, and the overall survival months were considered for the study (Figure 23).

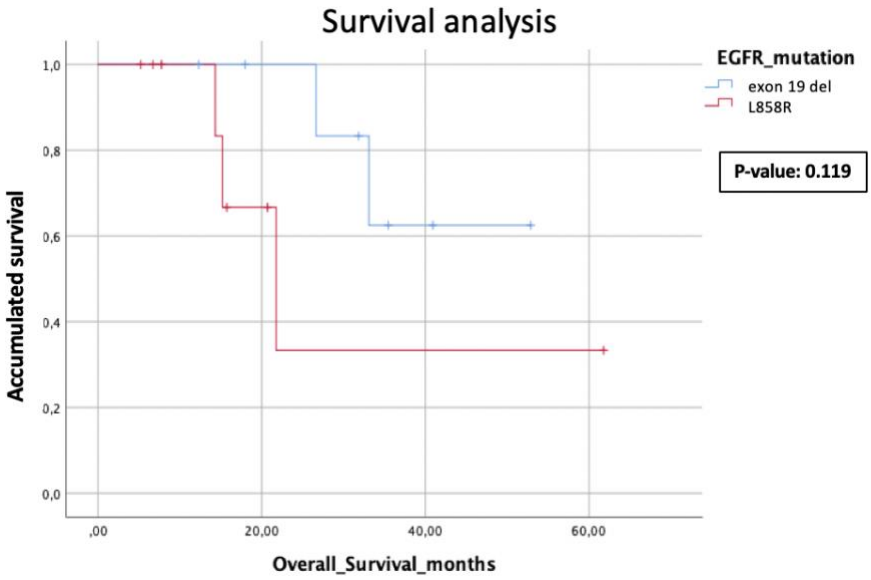


Figure 23. Kaplan – Meyer graphic for survival analysis dependent on the *EGFR* mutational status (L858R or exon 19 del).

The p-value obtained for this analysis was not significant (p-value = 0.119), although patients with exon 19 del mutation seem to have a longer overall survival than those with L858R mutation. This hypothesis has been demonstrated in other studies which correlate exon 19 del mutation with favorable overall survival (Choi et al., 2018, Renaud et al., 2018). Therefore, *EGFR* mutation subtype should be considered when making treatment decision or designing clinical trials for *EGFR* mutation-positive advanced NSCLC patients.

4.5. CLINICAL IMPLEMENTATION OF LIQUID BIOPSY FOR PATIENT MONITORING

In summary, a total of 159 tissue and liquid biopsies were analysed and compared to obtain the concordance between different techniques and evaluate the diagnostic/predictive information of liquid biopsies. Once the patients were treated with first-generation EGFR-TKIs, several blood samples were obtained to monitor the disease and evaluate their prognosis. 10 patients presented disease progression which was corroborate by an increasing of the MAF of their corresponding sensitizing mutations at 4-8 weeks after starting the treatment, being 8 of them (80%) also mutated in T790M. Those patients mutated in T790M develop a resistance to the EGR-TKIs administered and new treatment strategies had to be selected. The detection of this resistance mutation was observed in 5 patients (50%) prior to radiological progression which corroborates the predictive value of liquid biopsies. Moreover, patients who were treated with third-generation EGFR-TKIs after progression continued being monitored to predict future responses (Figure 24).

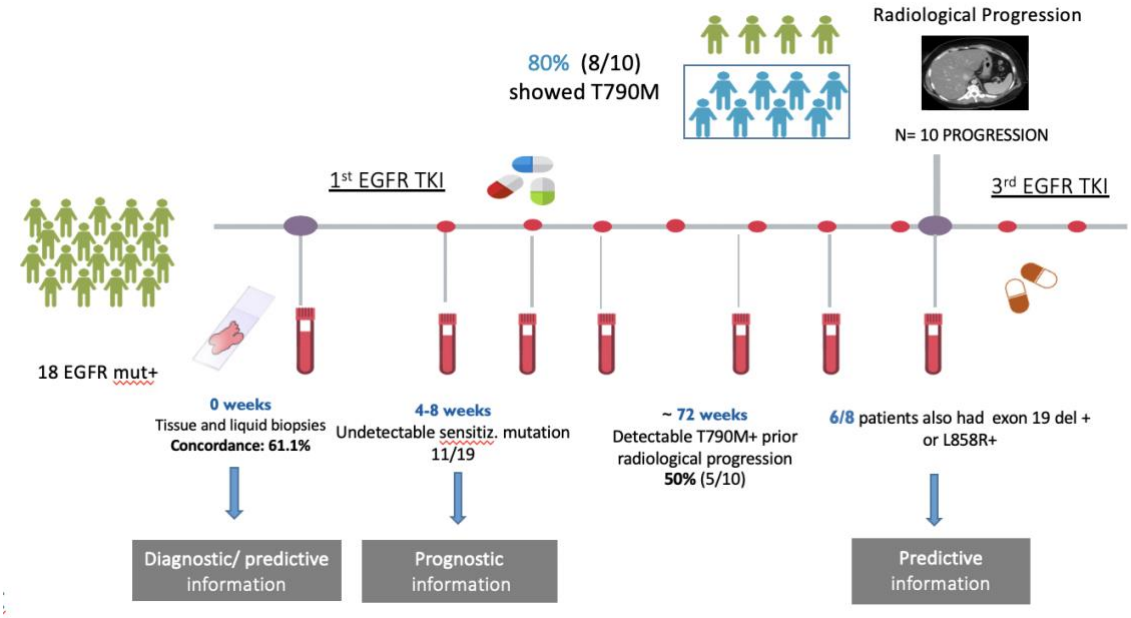


Figure 24. Our experience in the General Hospital of Valencia with 18 *EGFR* mutated patients monitoring during the last five years.

Altogether, it can be concluded that dPCR is a very sensitive technology for the analysis of ctDNA in *EGFR*-mutated advanced NSCLC patients. In this context, it represents the beginning of an innovative approach to molecular diagnostics of cancer, which has the potential to inform early detection of cancer, detect minimal residual disease, mirror the heterogeneity of tumor and track evolution of resistant disease. Therefore, the results obtaining using liquid biopsy as a routinely analysis may influence treatment decisions to have the best clinical management and ultimately improve patient survival.

5. CONCLUSIONS

1. The results presented confirm that the analysis of ctDNA by BEAMing and QuantStudio™ digital PCR platforms in advanced NSCLC are sensitive technology for the analysis of mutational status of *EGFR*, including L858R, exon 19 deletion and T790M mutations.

2. The relative high concordance of the *EGFR* mutation results between plasma and tissue is demonstrated (61.1%), which shows that the *EGFR* mutation test in blood is a viable alternative to tissue-based mutation analysis using highly sensitive dPCR methods.

3. Monitoring of the *EGFR* mutational status and allelic fraction in LB during EGFR-TKI offer the opportunity to systematically track genomic evolution, assess patient's prognosis and detect disease recurrence earlier. Analysis of ctDNA in plasma samples obtained before and after treatment can ultimately provide a global picture of the genetic alterations of a patient's tumor.

4. The existence of a relationship between the acquisition of the resistance mutation T790M and patient's prognosis. Knowledge of T790M status is therefore important both for the clinical care of these patients and for the optimal design and interpretation of clinical trials.

5. The periodic monitoring of patients by LB represents an innovative approach to molecular diagnosis of cancer, based on the systemic evaluation of the disease, being dPCR a very promising technique for monitoring EGFR-mutated NSCLC patients in clinical routine and as a surrogate of tissue biopsies.

6. REFERENCES

ABBOSH, C., BIRKBAK, N. J., WILSON, G. A., JAMAL-HANJANI, M., CONSTANTIN, T., SALARI, R., LE QUESNE, J., MOORE, D. A., VEERIAH, S., ROSENTHAL, R., MARAFIOTI, T., KIRKIZLAR, E., WATKINS, T. B. K., MCGRANAHAN, N., WARD, S., MARTINSON, L. & RILEY, J. (2017). 'Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution'. *Nature* 545:446–451.

ALBERG, A. J., & SAMET, J. M., (2003). Epidemiology of lung cancer. *Chest*, *123*(1), 21S-49S.

ALIX-PANABIERES C., SCHWARZENBACH H., PANTEL K. (2012) Circulating tumor cells and circulating tumor DNA. *Annu Rev Med 2012;* 63:199–215.

ANTONIO, C. D., PASSARO, A., GORI, B., SIGNORE, E. DEL, MIGLIORINO, M. R., RICCIARDI, S., FULVI, A. & MARINIS, F. DE. (2014). 'Bone and brain metastasis in lung cancer : recent advances in therapeutic strategies':101–114.

ARAVANIS, A. M., LEE, M., KLAUSNER, R. D. & PARK, M. (2017). 'Commentary Next-Generation Sequencing of Circulating Tumor DNA for Early Cancer Detection'. *Cell* 168:571–574. Elsevier Inc.

BARBERIS, I., MARTINI, M., IAVARONE, F., ORSI, A., PAVLOVA, N. N., THOMPSON, C. B., MALAPELLE, U., PISAPIA, P., ROCCO, D., SMERAGLIO, R., DI SPIRITO, M., BELLEVICINE, C., TRONCONE, G., LENZ, C., STEFFEN, J. A., ENGELSBERG, A. & ALIX-PANABIÈRES, C. (2016). 'MicroRNA profiling associated with non-small cell lung cancer: next generation sequencing detection, experimental validation, and prognostic value'. *Chest* 10:56–63. Elsevier Inc.

BARTELS, S., PERSING, S., HASEMEIER, B., SCHIPPER, E., KREIPE, H. & LEHMANN, U. (2017). 'Molecular Analysis of Circulating Cell-Free DNA from Lung Cancer Patients in Routine Laboratory Practice A Cross-Platform Comparison of Three Different Molecular Methods for Mutation Detection'. *The Journal of Molecular Diagnostics* 19:722–732. American Society for Investigative Pathology and the Association for Molecular Pathology.

BAUML, J. & LEVY, B. (2018). 'Clonal Hematopoiesis: A New Layer in the Liquid Biopsy Story in Lung Cancer'. *Clinical Cancer Research* 24:4352 LP – 4354.

BETHUNE, G., BETHUNE, D., RIDGWAY, N. & XU, Z. (2010). 'Epidermal growth factor receptor (EGFR) in lung cancer : an overview and update'. *The Journal of thoracic and cardiovascular surgery* 2:48–51.

BETTEGOWDA, C., SAUSEN, M., LEARY, R. J., KINDE, I., WANG, Y., AGRAWAL, N. & ... ... & ANTONARAKIS, E. S. (2014). 'Detection of Circulating Tumor DNA in Early and Late Malignancies'. *National Institute Health* 6.

BEZJAK, A., TU, D., SEYMOUR, L., CLARK, G., TRAJKOVIC, A., ZUKIN, M., AYOUB, J., LAGO, S., RIBEIRO, R. D. A., GEROGIANNI, A., CYJON, A., NOBLE, J., LABERGE, F., CHAN, R. T. T., FENTON, D., VON PAWEL, J., RECK, M. & SHEPHERD, F. A. (2006). 'Symptom improvement in lung cancer patients treated with erlotinib: Quality of life analysis of the National Cancer Institute of Canada clinical trials group study BR.21'. *Journal of Clinical Oncology* 24:3831–3837.

BIAN, X., JING, F., LI, G., FAN, X., JIA, C., ZHOU, H., ... & ZHAO, J. (2015). A microfluidic droplet digital PCR for simultaneous detection of pathogenic Escherichia coli O157 and Listeria monocytogenes. *Biosensors and Bioelectronics*, *74*, 770-777.

BRAY, F. (2018). 'Global Cancer Statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries'. *CA Cancer J Clin.*:394–424.

BRENNER, D. R., HUNG, R. J., TSAO, M.-S., SHEPHERD, F. A., JOHNSTON, M. R., NAROD, S., RUBENSTEIN, W. & MCLAUGHLIN, J. R. (2010). 'Lung cancer risk in never-smokers: a population-based case-control study of epidemiologic risk factors'. *BMC Cancer* 10:285. BioMed Central.

BYRNE, O. (2015). *Effect of afatinib on overall survival of patients with EGFR mutation-positive lung adenocarcinoma : outcomes of the two randomised Phase III trials LUX-Lung 3 and LUX-Lung 6*.

CALABUIG-FARIÑAS, S., JANTUS-LEWINTRE, E., HERREROS-POMARES, A. & CAMPS, C. (2016). 'Circulating tumor cells versus circulating tumor DNA in lung cancer-which one will win?' *Translational lung cancer research* 5:466–482. AME Publishing Company.

CARDENAL, F., CAMPS, C., MAJEM, M., LOPEZ-VIVANCO, G., ISLA, D., PROVENCIO, M., INSA, A., MASSUTI, B., GONZALEZ-LARRIBA, J. L., PAZ-ARES, L., BOVER, I., GARCIA-CAMPELO, R., MORENO, M. A., CATOT, S., ROLFO, C., REGUART, N., PALMERO, R., SÁNCHEZ, J. M., BASTUS, R., MAYO, C., PH, D., BERTRAN-ALAMILLO, J., MOLINA, M. A., PH, D., SANCHEZ, J. J., TARON, M. & PH, D. (2009). 'Screening for Epidermal Growth Factor Receptor Mutations in Lung Cancer'.

CHEN, Z., FILLMORE, C. M., HAMMERMAN, P. S., KIM, C. F., WONG, K. & PROGRAM, C. (2017). 'Non-small-cell lung cancers: a heterogeneous set of diseases'. *Nature Reviews Cancer* 14:535–546.

CHOI, Y. W., JEON, S. Y., JEONG, G. S., LEE, H. W., JEONG, S. H., KANG, S. Y., PARK, J. S., CHOI, J.-H., KOH, Y. W. & HAN, J. H. (2018). 'EGFR Exon 19 Deletion is Associated With Favorable Overall Survival After First-line Gefitinib Therapy in Advanced Non–Small Cell Lung Cancer Patients'. *American journal of clinical oncology* 41:385–390. Wolters Kluwer.

CLARK, J., COOLS, J. & GILLILAND, D. G. (2005). 'EGFR Inhibition in Non-Small Cell Lung Cancer : Resistance , Once Again , Rears Its Ugly Head' 2:195–197.

COLLINS, L. G., HAINES, C., PERKEL, R. & ENCK, R. E. (2007). 'Lung cancer: Diagnosis and management'. *American Family Physician* 75:56–63.

CONTE, D., VERRI, C., BORZI, C., SUATONI, P., PASTORINO, U., SOZZI, G. & FORTUNATO, O. (2015). 'Novel method to detect microRNAs using chip-based QuantStudio 3D digital PCR'. *BMC Genomics*:1–11. BMC Genomics.

CORRAL, M. C. J. & VIN, N. (2015). 'SEOM clinical guidelines for the treatment of non-small cell lung cancer (NSCLC) 2015':1020–1029.

CRINÒ, L., WEDER, W., VAN MEERBEECK, J. & FELIP, E. (2010). 'Early stage and locally advanced (non-metastatic) non-small-cell lung cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up'. *Annals of Oncology* 21.

CROWLEY, E., DI NICOLANTONIO, F., LOUPAKIS, F. & BARDELLI, A. (2013). 'Liquid biopsy: Monitoring cancer-genetics in the blood'. *Nature Reviews Clinical Oncology* 10:472–484. Nature Publishing Group.

DAY, E., DEAR, P. H. & MCCAUGHAN, F. (2013). 'Digital PCR strategies in the development and analysis of molecular biomarkers for personalized medicine'. *Methods* 59:101–107. Elsevier Inc.

DE LUCA, A., CAROTENUTO, A., RACHIGLIO, A., GALLO, M., MAIELLO, M. R., ALDINUCCI, D., ... & NORMANNO, N. (2008). The role of the EGFR signaling in tumor microenvironment. *Journal of cellular physiology*, *214*(3), 559-567.

DE MATTOS-ARRUDA, L., MAYOR, R., NG, C. K. Y., WEIGELT, B., MARTÍNEZ-RICARTE, F., TORREJON, D., OLIVEIRA, M., ARIAS, A., RAVENTOS, C., TANG, J., GUERINI-ROCCO, E., MARTÍNEZ-SAÉZ, E., LOIS, S., MARÍN, O., DE LA CRUZ, X., PISCUOGLIO, S., TOWERS, R., VIVANCOS, A., PEG, V., CAJAL, S. R. Y., CARLES, J., RODON, J., GONZÁLEZ-CAO, M., TABERNERO, J., FELIP, E., SAHUQUILLO, J., BERGER, M. F., CORTES, J., REIS-FILHO, J. S. & SEOANE, J. (2015). 'Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma'. *Nature Communications* 6:1–6.

DETTERBECK, F. C., DECAMP, M. M., KOHMAN, L. J. & SILVESTRI, G. A. (2003). 'Invasive staging: The guidelines'. *Chest* 123:167S-175S.

DIAZ, L. A. & BARDELLI, A. (2014). 'Liquid biopsies: Genotyping circulating tumor DNA'. *Journal of Clinical Oncology* 32:579–586.

FRIDMAN, W. H., PAGES, F., SAUTES-FRIDMAN, C., & GALON, J. (2012). The immune contexture in human tumours: impact on clinical outcome. *Nature Reviews Cancer*, *12*(4), 298.

GADZAR, A. F. (2010). 'Activating and resistance mutations of EGFR in non-small cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors'. *Oncogene* 28:1–14.

GARCÍA-CAMPELO, R., BERNABÉ, R., COBO, M., CORRAL, J., COVES, J., DÓMINE, M., NADAL, E., RODRIGUEZ-ABREU, D., VIÑOLAS, N. & MASSUTI, B. (2015). 'SEOM clinical guidelines for the treatment of non-small cell lung cancer (NSCLC) 2015'. *Clinical and Translational Oncology* 15:977–984.

GARCIA-MURILLAS, I., SCHIAVON, G., WEIGELT, B., NG, C., HREBIEN, S., CUTTS, R. J., CHEANG, M., OSIN, P., NERURKAR, A. & KOZAREWA, I. (2015). 'Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer'. *Science translational medicine* 7:302ra133-302ra133. American Association for the Advancement of Science.

GATELY, K., O'FLAHERTY, J., CAPPUZZO, F., PIRKER, R., KERR, K. & O'BYRNE, K. (2012). 'The role of the molecular footprint of EGFR in tailoring treatment decisions in NSCLC'. *Journal of Clinical Pathology* 65:1–7.

GRIDELLI, C., ROSSI, A., CARBONE, D. P., GUARIZE, J., KARACHALIOU, N., MOK, T., et al. (2015). Non-small-cell lung cancer. *Nature Reviews Disease Primers*, *1*, 15009

HANAHAN, D. & COUSSENS, L. M. (2012). 'Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment'. *Cancer Cell* 21:309–322. Elsevier Inc.

HANAHAN, D. & WEINBERG, R. A. (2011). 'Review Hallmarks of Cancer : The Next Generation'. *Cell* 144:646–674. Elsevier Inc.

HEUVERS, M. E., HEGMANS, J. P., STRICKER, B. H. & AERTS, J. G. (2012). 'Improving lung cancer survival; time to move on'. *BMC Pulmonary Medicine* 12:2–5.

HUDECOVA, I. (2015). 'Digital PCR analysis of circulating nucleic acids'. *Clinical Biochemistry* 48:948–956. The Canadian Society of Clinical Chemists.

HWANG, S. J., CHENG, L. S. C., LOZANO, G., AMOS, C. I., GU, X. & STRONG, L. C. (2003). 'Lung cancer risk in germline p53 mutation carriers: Association between an inherited cancer predisposition, cigarette smoking, and cancer risk'. *Human Genetics* 113:238–243.

INAMURA, K., NINOMIYA, H., ISHIKAWA, Y. & MATSUBARA, O. (2010). 'Is the epidermal growth factor receptor status in lung cancers reflected in clinicopathologic features?' *Archives of Pathology and Laboratory Medicine* 134:66–72.

INTERNATIONAL, T. & GENOME, C. (2010). 'International network of cancer genome projects' 464.

INUKAI, M., TOYOOKA, S., ITO, S., ASANO, H., ICHIHARA, S., SOH, J., SUEHISA, H., OUCHIDA, M., AOE, K., AOE, M. & KIURA, K. (2006). 'Presence of Epidermal Growth Factor Receptor Gene T790M Mutation as a Minor Clone in Non – Small Cell Lung Cancer':7854–7859.

JACKMAN, D. M., YEAP, B. Y., SEQUIST, L. V., LINDEMAN, N., HOLMES, A. J., JOSHI, V. A., BELL, D. W., HUBERMAN, M. S., HALMOS, B., RABIN, M. S., HABER, D. A., LYNCH, T. J., MEYERSON, M., JOHNSON, B. E. & JÄNNE, P. A. (2006). 'Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib'. *Clinical Cancer Research* 12:3908–3914.

JANTUS-LEWINTRE, E., USO, M., SANMARTIN, E. & CAMPS, C. (2012). 'Update on biomarkers for the detection of lung cancer'. *Lung Cancer: Targets and Therapy.* 3:21–29. Dove Press.

JEMAL, A., BRAY, F., CENTER, M. M., FERLAY, J., WARD, E., & FORMAN, D. (2011). Global cancer statistics. *CA: a cancer journal for clinicians*, *61*(2), 69-90.

KOSAKA, T., YATABE, Y., ENDOH, H., KUWANO, H., TAKAHASHI, T. & MITSUDOMI, T. (2004). 'Mutations of the Epidermal Growth Factor Receptor Gene in Lung Cancer'. *Cancer Research* 64:8919–8923.

LAURING, J. & PARK, B. H. (2011). 'BEAMing Sheds Light on Drug Resistance' 17:7508–7511.

LI, X. & ZHOU, C. (2017). 'Comparison of cross-platform technologies for EGFR T790M testing in patients with non-small cell lung cancer' 8:100801–100818.

LIVAK, K.J., FLOOD, S.J.A., MARMARO, J., AND MULLAH, K.B., inventors; Applied Biosystems (Foster City, CA), assignee. 2 Mar. 1999. Hybridization assay using self-quenching fluorescence probe. United States ptent 5,876,930.

MA, J., LI, N., GUARNERA, M. & JIANG, F. (2013). 'Quantification of plasma miRNAs by digital PCR for cancer diagnosis'. *Biomarker Insights* 8:127–136.

MALAPELLE, U., PISAPIA, P., ROCCO, D., SMERAGLIO, R., DI SPIRITO, M., BELLEVICINE, C. & TRONCONE, G. (2016). 'Next generation sequencing techniques in liquid biopsy: focus on non-small cell lung cancer patients'. *Translational Lung Cancer Research* 5:505–510.

MAO, X., ZHANG, Z., ZHENG, X., XIE, F., DUAN, F., JIANG, L., CHUAI, S., HAN-ZHANG, H., HAN, B. & SUN, J. (2017). 'Capture-Based Targeted Ultradeep Sequencing in Paired Tissue and Plasma Samples Demonstrates Differential Subclonal ctDNA-Releasing Capability in Advanced Lung Cancer'. *Journal of Thoracic Oncology* 12:663–672. Elsevier Inc.

MARCHETTI, A., FELICIONI, L., MALATESTA, S., SCIARROTTA, M. G., GUETTI, L., CHELLA, A., VIOLA, P., PULLARA, C., MUCILLI, F. & BUTTITTA, F. (2011). 'Clinical features and outcome of patients with non-small-cell lung cancer harboring BRAF mutations'. *Journal of Clinical Oncology* 29:3574–3579.

MAZZONE P., JAIN P., ARROLIGA A.C., MATTHAY R.A. (2002) Bronchoscopy and needle biopsy techniques for diagnosis and staging of lung cancer. *Clinics in chest medicine*, *23*(1), 137-58.

MIRSADRAEE, S., OSWAL, D., ALIZADEH, Y., CAULO, A. & VAN BEEK, E. (2012). 'The 7th lung cancer TNM classification and staging system: Review of the changes and implications'. *World Journal of Radiology* 4:128–34. Baishideng Publishing Group Inc.

MITSUDOMI, T. & YATABE, Y. (2010). 'Epidermal growth factor receptor in relation to tumor development : EGFR gene and cancer' 277:301–308.

MOGI, A. & KUWANO, H. (2011). 'TP53 Mutations in Nonsmall Cell Lung Cancer'. *Journal of Biomedicine and Biotechnology* 2011:1–9.

MOK, T. S., WU, Y.-L., AHN, M.-J., GARASSINO, M. C., KIM, H. R., RAMALINGAM, S. S., SHEPHERD, F. A., HE, Y., AKAMATSU, H., THEELEN, W. S. M. E., LEE, C. K., SEBASTIAN, M., TEMPLETON, A., MANN, H., MAROTTI, M., GHIORGHIU, S. & PAPADIMITRAKOPOULOU, V. A. (2016). 'Osimertinib or Platinum–Pemetrexed in EGFR T790M–Positive Lung Cancer'. *New England Journal of Medicine* 376:629–640. Massachusetts Medical Society.

LA MONICA, S., CRETELLA, D., BONELLI, M., FUMAROLA, C., CAVAZZONI, A., DIGIACOMO, G., FLAMMINI, L., BAROCELLI, E., MINARI, R., NALDI, N., PETRONINI, P. G., TISEO, M. & ALFIERI, R. (2017). 'Trastuzumab emtansine delays and overcomes resistance to the third-generation EGFR-TKI osimertinib in NSCLC EGFR mutated cell lines'. *Journal of Experimental & Clinical Cancer Research* 36:174.

MORGILLO, F., MARIA, C., CORTE, D., FASANO, M. & CIARDIELLO, F. (2016). 'Mechanisms of resistance to EGFR- targeted drugs : lung cancer':1–9.

MORIYA, T., TAKIGUCHI, Y., TABETA, H., WATANABE, R., KIMURA, H., NAGAO, K. & KURIYAMA, T. (2000). 'Controlling malignant pericardial effusion by intrapericardial carboplatin administration in patients with primary non-small-cell lung cancer'. *Nature* 83:858–862.

NATIONAL CANCER INSTITUTE Non-Small Cell Lung Cancer Treatment (PDQ®)- Healt Professional Version <https://www.cancer.gov/types/lung/hp/non-small-cell-lung-treatment-pdq> [Date Accessed: January 29, 2019]

NEAL, J. W., DAHLBERG, S. E., GRANT, S., WAKELEE, H. A. & AISNER, S. C. (2017). 'HHS Public Access' 17:1661–1671.

NOVELLO, S., BARLESI, F., CALIFANO, R., CUFER, T., EKMAN, S., LEVRA, M. G., KERR, K., POPAT, S., RECK, M., SENAN, S., SIMO, G. V, VANSTEENKISTE, J., PETERS, S. & COMMITTEE, G. (2016). 'clinical practice guidelines Metastatic non-small-cell lung cancer : ESMO Clinical Practice Guidelines for diagnosis , treatment and clinical practice guidelines' 27.

OXNARD, G. R., THRESS, K. S., ALDEN, R. S., LAWRANCE, R., PAWELETZ, C. P., CANTARINI, M., YANG, J. C. H., BARRETT, J. C. & JÄNNE, P. A. (2016). 'Association between plasma genotyping and outcomes of treatment with osimertinib (AZD9291) in advanced non-small-cell lung cancer'. *Journal of Clinical Oncology* 34:3375–3382.

OYA, Y., YOSHIDA, T., KURODA, H., SHIMIZU, J., HORIO, Y., SAKAO, Y., INABA, Y., HIDA, T. & YATABE, Y. (2017). 'Association between EGFR T790M status and progression patterns during initial EGFR-TKI treatment in patients harboring EGFR mutation'. *Clinical lung cancer* 18:698–705. Elsevier.

PAO, WILLIAM; CHMIELECKI, J. (2010). 'NIH Public Access' 10:760–774.

PARKIN, D. M. (2011). '14. Cancers attributable to occupational exposures in the UK in 2010.' *British journal of cancer*:S70-2. Nature Publishing Group.

PAVLOVA, N. N. & THOMPSON, C. B. (2016). 'The Emerging Hallmarks of Cancer Metabolism'. *Cell Metabolism* 23:27–47. Elsevier Inc.

PIETRAS, K. & ÖSTMAN, A. (2010). 'Hallmarks of cancer: Interactions with the tumor stroma'. *Experimental Cell Research* 316:1324–1331. Elsevier Inc.

PLANCHARD, D., LORIOT, Y., ANDRÉ, F., GOBERT, A., AUGER, N., LACROIX, L. & SORIA, J. C. (2015). 'EGFR-independent mechanisms of acquired resistance to AZD9291 in EGFR T790M-positive NSCLC patients':2073–2078.

PLANCHARD, D., POPAT, S., KERR, K., NOVELLO, S., SMIT, E. F., FAIVRE-FINN, C., MOK, T. S., RECK, M., VAN SCHIL, P. E., HELLMANN, M. D. & PETERS, S. (2018a). 'Metastatic non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up'. *Annals of Oncology* 29:iv192–iv237.

PLANCHARD, D., POPAT, S., KERR, K., NOVELLO, S., SMIT, E. F., FAIVRE-FINN, C., MOK, T. S., RECK, M., VAN SCHIL, P. E., HELLMANN, M. D. & PETERS, S. (2018b). 'Metastatic non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†'. *Annals of Oncology* 29:iv192–iv237. Oxford University Press.

POHL, G. & SHIH, I. (2004). 'Principle and applications of digital PCR'. *Expert Rev. Mol. Diagn* 4:41–47.

PROVENCIO, M., TORRENTE, M., CALVO, V., PÉREZ-CALLEJO, D., G., L., F. & F., ... & BUENO, C. (2017). 'Prognostic value of quantitative ctDNA levels in non small cell lung cancer patients'.

QI, Z. H., XU, H. X., ZHANG, S. R., XU, J. Z., LI, S., GAO, H. L., JIN, W., WANG, W. Q., WU, C. T., NI, Q. X., YU, X. J. & LIU, L. (2018). 'The significance of liquid biopsy in pancreatic cancer'. *Journal of Cancer* 9:3417–3426.

RENAUD, S., SEITLINGER, J., GUERRERA, F., REEB, J., BEAU-FALLER, M., VOEGELI, A.-C., SIAT, J., CLÉMENT-DUCHÊNE, C., TIOTIU, A. & SANTELMO, N. (2018). 'Prognostic value of Exon 19 versus 21 EGFR mutations varies according to disease stage in surgically resected non-small cell lung cancer adenocarcinoma'. *Annals of surgical oncology* 25:1069–1078. Springer.

RICHARDSON, A. L. & IGLEHART, J. D. (2012). 'BEAMing Up Personalized Medicine : Mutation Detection in Blood':3209–3212.

ROLFO, C., MACK, P. C., SCAGLIOTTI, G. V, BAAS, P., BARLESI, F., BIVONA, T. G., HERBST, R. S., MOK, T. S., PELED, N., PIRKER, R., RAEZ, L. E., RECK, M., RIESS, J. W., SEQUIST, L. V, SHEPHERD, F. A., SHOLL, L. M., TAN, D. S. W., WAKELEE, H. A., WISTUBA, I. I., WYNES, M. W., CARBONE, D. P., HIRSCH, F. R. & GANDARA, D. R. (2018). 'Liquid Biopsy for Advanced Non-Small Cell Lung Cancer (NSCLC): A Statement Paper from the IASLC'. *Journal of Thoracic Oncology* 13:1248–1268. Elsevier.

SCOCCIANTI, C., VESIN, A., MARTEL, G., OLIVIER, M., BRAMBILLA, E., TIMSIT, J. F., TAVECCHIO, L., BRAMBILLA, C., FIELD, J. K., HAINAUT, P., MARTINET, Y., THUNNISSEN, E., SNIJDERS, P., SOZZI, G., RISCH, A., ELBORN, S., MONTUENGA, L. M., O'BYRNE, K., HARRISON, D. J. & NIKLINSKI, J. (2012). 'Prognostic value of TP53, KRAS and EGFR mutations in nonsmall cell lung cancer: The EUELC cohort'. *European Respiratory Journal* 40:177–184.

SHARMA, S. V, BELL, D. W., SETTLEMAN, J. & HABER, D. A. (2007). 'Epidermal growth factor receptor mutations in lung cancer' 7:169–181.

SHEPHERD, F. A., CROWLEY, J., VAN HOUTTE, P., POSTMUS, P. E., CARNEY, D., CHANSKY, K., SHAIKH, Z. & GOLDSTRAW, P. (2007). 'The International Association for the Study of Lung Cancer lung cancer staging project: proposals regarding the clinical staging of small cell lung cancer in the forthcoming (seventh) edition of the tumor, node, metastasis classification for lung cancer.' *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* 2:1067–1077. United States.

SHIGEMATSU, H., LIN, L., TAKAHASHI, T., NOMURA, M., SUZUKI, M., WISTUBA, I. I., FONG, K. M., LEE, H., TOYOOKA, S., SHIMIZU, N., FUJISAWA, T., FENG, Z., ROTH, J. A., HERZ, J., MINNA, J. D. & GAZDAR, A. F. (2005). 'Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers'. *Journal of the National Cancer Institute* 97:339–346.

SIFAKI-PISTOLLA, D., LIONIS, C., KOINIS, F., GEORGOULIAS, V., & TZANAKIS, N. (2017). Lung cancer and annual mean exposure to outdoor air pollution in Crete, Greece. *European Journal of Cancer Prevention*, *26*, S208-S214.

SOCIEDAD ESPAÑOLA DE ONCOLOGÍA MÉDICA (SEOM). (2018). *Las cifras del cáncer en España 2018*. <https://seom.org/seomcms/images/stories/recursos/Las_Cifras_del_cancer_en_Espana2018.pdf> [Date Accessed: January 24, 2019]

SOCINSKI, M. A. & MORRIS, D. E. (2003). 'Chemotherapeutic Management of Stage IV Non-small Cell Lung Cancer *'.

SRIVASTAVA, M. K., BOSCH, J. J., WILSON, A. L., EDELMAN, M. J. & OSTRAND-ROSENBERG, S. (2010). 'MHC II lung cancer vaccines prime and boost tumor-specific CD4+T cells that cross-react with multiple histologic subtypes of nonsmall cell lung cancer cells'. *International Journal of Cancer* 127:2612–2621.

STROUN, M., MAURICE, P., VASIOUKHIN, V., LYAUTEY, J., LEDERREY, C., LEFORT, F., ROSSIER, A., CHEN, X. Q. & ANKER, P. (2006). 'The Origin and Mechanism of Circulating DNA'. *Annals of the New York Academy of Sciences* 906:161–168.

SYSMEX INOSTICS BEAMing Digital PCR < https://www.sysmex-inostics.com/science-technologies/beaming-digital-pcr.html > [Date Accessed: April 26, 2019].

THERMO FISHER SCIENTIFIC Product Bulletin QuantStudio ™ 3D Digital PCR System <https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1502-PJ8965-CO126174-Revise-QuantStudio-3D-Product-Bulletin-for-Branding-Changes-and-New-SKUs-Global-FLR.pdf> [Date Accessed: February 15, 2019].

THRESS, K. S., BRANT, R., CARR, T. H., DEARDEN, S., JENKINS, S., BROWN, H., HAMMETT, T., CANTARINI, M. & BARRETT, J. C. (2015). 'EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross-platform comparison of leading technologies to support the clinical development of AZD9291'. *Lung Cancer* 90:509–515. Elsevier Ireland Ltd.

TL, W. (2008). 'The tumor microenvironment and its role in promoting tumor growth. Oncogene'. *Oncogene* 27:5904–5912.

TOYOOKA, S., TSUDA, T. & GAZDAR, A. F. (2003). 'The TP53 gene, tobacco exposure, and lung cancer'. *Human Mutation* 21:229–239.

UCHIDA, J., KATO, K., KUKITA, Y., KUMAGAI, T., NISHINO, K. & DAGA, H. (2015). 'Diagnostic Accuracy of Noninvasive Genotyping of EGFR in Lung Cancer Patients by Deep Sequencing of Plasma Cell-Free DNA BACKGROUND : METHODS ': 1196:1191–1196.

V, N., J., Z., M., A. & M., J. (2013). 'Afatinib: Emerging next-generation tyrosine kinase inhibitor for NSCLC'. *OncoTargets and Therapy* 6:135–143.

VANDERLAAN, P. A., RANGACHARI, D., MOCKUS, S. M., SPOTLOW, V., REDDI, H. V, MALCOLM, J., HUBERMAN, M. S., JOSEPH, L. J., KOBAYASHI, S. S. & COSTA, D. B. (2017). 'Mutations in TP53, PIK3CA, PTEN and other genes in EGFR mutated lung cancers: Correlation with clinical outcomes'. *Lung cancer (Amsterdam, Netherlands)* 106:17–21.

WO, H., HE, J., ZHAO, Y., YU, H., CHEN, F. & YI, H. (2018). 'J o u r n a l o f C a n c e r The Efficacy and Toxicity of Gefitinib in Treating Non-small Cell Lung Cancer : A Meta-analysis of 19 Randomized Clinical Trials' 9.

YANG, J. C., AHN, M. & HORN, L. (2015). 'EGFR Gene Mutations'. Pp. 33–42 *IASLC ATLAS OF EGFR TESTING IN LUNG CANCER*.

YI, X., MA, J., GUAN, Y., CHEN, R., YANG, L. & XIA, X. (2017). 'The feasibility of using mutation detection in ctDNA to assess tumor dynamics' 2647:2642–2647.

YUN, C., BOGGON, T. J., LI, Y., WOO, M. S., GREULICH, H., MEYERSON, M. & ECK, M. J. (2007). 'Article Structures of Lung Cancer-Derived EGFR Mutants and Inhibitor Complexes : Mechanism of Activation and Insights into Differential Inhibitor Sensitivity':217–227.

YUN, C., MENGWASSER, K. E., TOMS, A. V, WOO, M. S., GREULICH, H., WONG, K., MEYERSON, M. & ECK, M. J. (2008). 'The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP'.

YUNG, T. K. F., CHAN, K. C. A., MOK, T. S. K., TONG, J., TO, K. F. & LO, Y. M. D. (2009). 'Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients'. *Clinical Cancer Research* 15:2076–2084.

ZHENG, G., TSENG, L. H., CHEN, G., HALEY, L., ILLEI, P., GOCKE, C. D., ESHLEMAN, J. R. & LIN, M. T. (2015). 'Clinical detection and categorization of uncommon and concomitant mutations involving BRAF'. *BMC Cancer* 15:1–10. BMC Cancer.

7. APPENDICES

7.1. Appendix I. Supplementary table.

Supplementary Table 1. Monitoring of 18 patients with advanced NSCLC.

Date	26/5/17	29/6/17	17/7/17	28/8/17	23/10/17	27/11/17	19/4/18	21/6/18	23/8/18	17/10/18	13/11/18	5/12/18
Patient 1	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	7th Det	8th Det	9th Det	10th Det	11th Det	12th Det
	WT	L858R	L858R	L858R	WT	WT	WT	L858R+T790M	L858R+T790M	L858R+T790M	WT	WT
Treatment	**SURGICAL RESECTION**				**GEFITINIB (1st line)**			**OSIMERTINIB (2nd line)**				

Date	7/8/18	20/9/18	8/11/18	13/12/18	11/2/19	4/4/19
Patient 2	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det
	L858R	L858R	WT	L858R+T790M	L858R+T790M	L858R+T790M
Treatment	**After CHEMO**	**GEFITINIB (1st line)**				

Date	9/11/17	15/3/18	4/10/18	29/11/18	21/3/19
Patient 3	1st Det	2nd Det	3rd Det	4th Det	5th Det
	exon 19 del	WT	WT	WT	WT
Treatment	**S. RESECTION**	**GEFITINIB (1st line)**			

Date	22/3/18	21/6/18	18/9/18	18/10/18	9/11/18	24/1/19	28/3/19
Patient 4	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	
	L858R	WT	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M	L858R+T790M
Treatment	**S. RESECTION**	**GEFITINIB (1st line)**					

Date	1/12/17	19/4/18	11/2/19	9/5/19
Patient 5	1st Det	2nd Det	3rd Det	4th Det
	L858R+T790M	L858R	L858R+T790M	L858R+T790M
Treatment	**GEFITINIB (1st line)**		**OSIMERTINIB (2nd line)**	

Date	19/10/17	4/12/17	1/3/18	19/4/18	15/6/18	15/2/19
Patient 6	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det
	L858R	WT	WT	L858R	L858R	L858R
Treatment	**After CHEMO**	**GEFITINIB (1st line)**				**CARBO**

Date	10/5/17	1/3/18	19/4/18	10/5/18	25/10/18	31/1/19	4/4/19
Patient 7	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	7th Det
	exon 19 del	WT	WT	WT	WT	WT	WT
Treatment	**After CHEMO**	**GEFITINIB (1st line)**					

Date	23/10/17	21/12/17	5/7/18	22/11/18	28/2/19
Patient 8	1st Det	2nd Det	3rd Det	4th Det	5th Det
	WT	WT	WT	WT	WT
Treatment	**GEFITINIB (1st line)**		**ERLOTINIB (2nd line)**		

Date	28/11/16	19/6/17	4/9/17	13/12/17	7/3/18	7/6/18
Patient 9	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det
	WT	WT	exon 19 del	exon 19 del	exon 19 del	exon 19 del
Treatment	**GEFITINIB (1st line)**					

Date	22/7/14	4/9/14	29/4/15	20/11/15	17/12/15	14/4/16
Patient 10	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det
	L858R	WT	WT	L858R+T790M	L858R+T790M	WT
Treatment	**S. RESECTION**	**GEFITINIB (1st line)**				

Date	17/10/14	11/2/15	10/3/16	25/7/16
Patient 11	1st Det	2nd Det	3rd Det	4th Det
	WT	exon 19 del	exon 19 del	exon 19 del
Treatment	**S. RESECTION**	**GEFITINIB (1st line)**		

Date	11/12/15	7/3/16	11/4/16	14/11/16	1/12/16	26/1/17
Patient 12	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det
	L858R	WT	L858R	L858R+T790M	L858R+T790M	L858R+T790M
Treatment	**S. RESECTION**	**GEFITINIB (1st line)**				

Date	23/2/17	5/4/17	8/6/17	28/7/17	21/12/17	15/2/18	11/5/18	7/6/18	11/10/18	8/11/18	8/2/19
Patient 13	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	7th Det	8th Det	9th Det	10th Det	11th Det
	L858R	L858R	L858R	WT	L858R+T790M	L858R+T790M	L858R+T790M	L858R	L858R	L858R	L858R
Treatment	**S. RESECTION**	**ERLOTINIB (1st line)**					**OSIMERTINIB (2nd line)**				

Date	8/2/18	24/5/18	8/11/18	4/4/19
Patient 14	1st Det	2nd Det	3rd Det	4th Det
	WT	WT	WT	WT
Treatment	**GEFITINIB (2nd line)**			

Date	1/7/14	31/7/14	20/10/16	29/5/17	31/7/17	2/10/17	20/11/17	4/12/17	3/5/18	14/6/18	7/2/19
Patient 15	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	7th Det	8th Det	9th Det	10th Det	11th Det
	exon 19 del	WT	WT	exon 19 del+T790M	exon 19 del+T790M	WT	WT	WT	WT	WT	exon 19 del
Treatment	**ERLOTINIB (1st line)**				**OSIMERTINIB (2nd line)**						

Date	16/2/17	30/3/17	29/6/17	9/11/17	7/2/18	9/5/18	19/7/18	8/8/18	11/10/18	2/11/18	11/1/19	4/4/19
Patient 16	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	7th Det	8th Det	9th Det	10th Det	11th Det	12th Det
	L858R	WT	WT	WT	L858R+T790M	L858R+T790M	L858R	L858R+T790M	WT	WT	WT	WT
Treatment	**S. RESECTION**	**ERLOTINIB (1st line)**					**BEVACIZUMAB/OSIMERTINIB (2nd line)**					

Date	1/4/15	3/12/15	23/3/16	29/5/17	16/11/16	12/12/16	12/1/17	2/3/17	14/5/17	15/6/17
Patient 17	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	7th Det	8th Det	9th Det	10th Det
	WT	T790M	T790M	T790M	WT	WT	WT	WT	WT	WT
Treatment	**ERLOTINIB (1st line)**			**OSIMERTINIB (2nd line)**						

Date	24/4/15	20/11/15	17/12/15	29/5/17	17/7/17	30/10/17	20/11/17	29/12/17	8/2/18	1/3/18	19/11/18	28/2/19
Patient 18	1st Det	2nd Det	3rd Det	4th Det	5th Det	6th Det	7th Det	8th Det	9th Det	10th Det	11th Det	12th Det
	exon 19 del	WT	WT	WT	exon 19 del	WT	WT	exon 19 del	WT	WT	WT	exon 19 del
Treatment	**S. RESECTION**	**AFATINIB (1st line)**			**OSIMERTINIB (2nd line)**							