Universidade de Lisboa

Faculdade de Farmácia

Advanced therapies for precision medicine in oncology

Non-small cell lung cancer

Mariana Alves Mimoso

Monografia orientada pela Professora Doutora Helena Isabel Fialho Florindo Roque Ferreira, Professora Associada com Agregação e coorientada pela Doutora Rita Acúrcio, Investigadora.

Mestrado Integrado em Ciências Farmacêuticas

2022

To my father. Hope you are proud.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

O cancro é um problema mundial e, apesar dos avanços da ciência e medicina na compreensão da sua biologia e genética, é a segunda causa de morte. Durante muito tempo, o seu tratamento baseou-se na cirurgia, radioterapia e quimioterapia. Embora estas terapias tenham apresentado resultados positivos, apresenta taxas de sucesso variáveis devido à falta de especificidade e toxicidade sistémica inerente.

A medicina de precisão introduz uma mudança de paradigma no campo médico, mostrando-se promissora no diagnóstico atempado, bem como na melhoria das condições de vida dos doentes com cancro. O objetivo é avaliar dados genéticos, estilo de vida e ambientais do paciente, com recurso a tecnologia de ponta, como testes moleculares e sequenciamento de última geração, para fornecer um diagnóstico preciso num estadio inicial, bem como um tratamento apropriado. Assim, a medicina de precisão pode permitir a prevenção de doenças e, ao mesmo tempo, reduzir os custos e efeitos colaterais da terapêutica.

Atualmente, o campo com maior pesquisa e aplicação em medicina de precisão, é a oncologia. O cancro do pulmão é um exemplo proeminente do sucesso desta inovação no tratamento de tumores sólidos malignos. O cancro de pulmão é um grande problema de saúde pública, sendo a principal causa mundial de morte relacionada com o cancro. Isto deve-se a um diagnóstico em estadios avançados, em consequência da ausência de sintomas na fase inicial da doença, o que a torna muitas vezes incurável. Assim, a realização do perfil molecular é uma ferramenta ideal para o diagnóstico e deteção atempados. A caracterização molecular abrangente do cancro de pulmão expandiu a nossa perceção das origens celulares e vias moleculares afetadas em cada um dos subtipos. Além disso, muitas das alterações genéticas encontradas representam potenciais alvos terapêuticos (biomarcadores) para os quais novos fármacos estão em constante desenvolvimento.

Esta monografia pretende dar uma visão geral da atual medicina de precisão no cancro, bem como as suas perspetivas futuras. Foi dado destaque ao cancro do pulmão, mais precisamente ao subtipo de cancro do pulmão de células não pequenas, devido à sua alta incidência e mortalidade.

Palavras-chave: Medicina de Precisão; Cancro do Pulmão de Células Não Pequenas; Biomarcadores; Sequenciamento de Última Geração.

Abstract

Cancer is a worldwide problem, and despite tremendous advances in science and medicine in the understanding of its biology and genetics, it is still a major cause of death. For a long time, surgery, radiotherapy, and chemotherapy were the most used approaches for cancer treatment. Even though these therapies have shown positive outcomes, variable success rates have been obtained due to their lack of specificity and inherent systemic toxicity.

Precision medicine is a new, paradigm-shift approach in medical field that shows promise for improving many aspects of healthcare, both in terms of diagnosis and treatment of diseases. The goal of precision medicine is to use cutting-edge healthcare resources, such as molecular tests and next-generation sequencing to decipher patient's genomic, lifestyle, and environmental data, and thereby identify a precise diagnosis at an earlier stage, as wells as an appropriate treatment. In this way, precision medicine may allow the prevention of disease while also reducing the costs and side effects of therapy.

Currently, oncology is the field with major research and application of precision medicine. A prominent example of the success of this innovation in treating solid tumour malignancies, is lung cancer. Lung cancer is a major public health concern, being the leading cause of cancer related mortality worldwide. The reason for this is the diagnosis at an advance stage, due to the absence of symptoms at early stages, which makes the disease often incurable. As such, molecular profiling is the ideal tool for early diagnosis and detection. Comprehensive molecular characterization of lung cancer has expanded our understanding of the cellular origins and molecular pathways affected in each of the sub-types. Furthermore, many of these genetic alterations represent potential therapeutic targets (biomarkers) for which novel drugs are constantly under development.

This monograph aims to give an overview of cancer precision medicine current state and future perspectives. A special focus was given to lung cancer, more precisely to the non-small cell lung cancer subtype, due to its high incidence and mortality.

Keywords: Precision Medicine; Non-Small Cell Lung Cancer; Biomarkers; Next-Generation Sequencing

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Abbreviations

ALCL – Anaplastic Large-Cell Lymphoma

ALK – Anaplastic Lymphoma Kinase

ATP – Adenosine Triphosphate

BC – Before Christ

BRAF – V-Raf Murine Sarcoma Viral Oncogene Homolog B

BRCA1 – Breast Cancer Gene 1

BRCA2 – Breast Cancer Gene 2

ctDNA – Circulating Tumour DNA

CTLA-4 – Cytotoxic T-Lymphocyte-Associated Protein 4

DNA – Deoxyribonucleic Acid

EGFR – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

EMA – European Medicines Agency

EML4 – Echinoderm Microtubule-Associated Protein-Like 4

ESMO – European Society for Medical Oncology

Fc – Fragment Crystallizable

FDA – Food and Drug Administration

FISH – Fluorescence *in-situ* hybridization

GEF – Guanine Nucleotide Exchange Factor

GDP – Guanosine Diphosphate

GTPases – Guanosine Triphosphate Hydrolyses

HER2 – Human Epidermal Growth Factor Receptor 2

HRAS – Harvey Rat Sarcoma Viral Oncogene Homolog

IARC – International Agency for Research on Cancer

ICH – Immunohistochemistry

IgG – Immunoglobulin G

KRAS – Kirsten Rat Sarcoma Viral Oncogene Homolog

KRAS G12C – Kirsten Rat Sarcoma Viral Oncogene Homolog Glycine 12 to Cysteine

MAbs – Monoclonal Antibodies

MAPK/ERK – Mitogen-Activated Protein Kinase/ Extracellular Signal-Regulated Kinase

MDM2 – Mouse Double Minute 2

MET – Mesenchymal Epithelial Transition Factor Receptor

NGS – Next-Generation Sequencing

NPM – Nucleophosmin

NRAS – Neuroblastoma RAS Viral Oncogene Homolog

NTRK – Neurotrophic Tyrosine Receptor Kinase

NSCLC – Non-Small Cell Lung Cancer

PD1 – Programmed Cell Death 1

PD-L1 – Programmed Death-Ligand 1

PIK3CA – Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PI3K/AKT/mTOR – Phosphoinositide 3-Kinase - Protein Kinase B - Mammalian Target of Rapamycin

PLC – Phospholipase C

PTB – Phosphotyrosine-Binding

RET – Rearranged During Transfection

RNA – Ribonucleic Acid

ROS1 – ROS Proto-Oncogene 1 Receptor Tyrosine Kinase

RTK – Receptor Tyrosine Kinases

SCLC – Small Cell Lung Cancer

SH2 – Src Homology 2

SNS – Serviço Nacional de Saúde

TK - Tyrosine Kinase

TKI - Tyrosine Kinase Inhibitor

TTF1 – Thyroid Transcription Factor 1

TP53 – Tumour-Suppressor Gene Tumour Protein p53

TRK – Tropomyosin Receptor Kinase

VEGFR – Vascular Endothelial Growth Factor Receptor

WHO – World Health Organization

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Table Index

I - Introduction

Cancer is our society's greatest medical challenge since it is affecting people of all ages, genders, and social status, all over the world. Its incidence, as well as its mortality rate, continues to rise worldwide, accounting for nearly 10 million deaths in 2020. According to the International Agency for Research on Cancer (IARC), the number of cancer cases will rise from 19.2 million to just over 30 million in less than 20 years. Considering these numbers, it is safe to predict that it might affect all of us, either if we are the ones facing it or know someone who does (1,2).

Cancer is a complex set of genetic diseases that can affect any organ of the body, characterised by uncontrolled proliferation, invasion, and metastasis, as well as by the capacity to evade cell death and immune system surveillance. There are a variety of features that might contribute to the development of cancer, such as age, environment, diet and hereditary. Lung cancer is one of the types of cancer with the highest incidence and it remains the leading cause of cancer death, with an estimated 1.8 million deaths in 2021 (3).

There are three types of traditional cancer treatments: surgery, chemotherapy, and radiotherapy. These therapies assume that every patient with a certain type of cancer will respond the same way to the treatment. However, cancer has shown otherwise, as it has a significant genetic component, which favours the occurrence of several genetic mutations during tumour's growth, producing cell markers with unique characteristics, depending on the exact location of the tumour and its staging. This makes traditional therapies less effective since they are very generalized. Nonetheless, despite this variability and several drawbacks, not only in terms of potential side effects, but also in terms of the specificity and effectivity in eradicating all tumoral cells, most types of tumours are still being treated with traditional therapies. In an attempt to address these challenges, the search for novel and safer therapeutic alternatives has been encouraged, especially given the complexity and aggressiveness of cancer. As a result, novel approaches have been developed to specifically target cancer cells and hence, promote health by influencing illness progression or treatment response, improving patients' quality of life by minimizing side effects and improve survival rate. (4–7).

Aside from novel target therapies, researchers are interested in developing new methodologies that would grant an early and individualized diagnosis, resulting in a better chance of a

favourable response to the appropriate treatment and, consequently, in lower morbidity and treatment costs.

Precision medicine is a new, game-changing approach that, in association with the technological progress, seeks to enhance illness prevention, early diagnosis, and treatment response by considering each person's genetic diversity, environment, and lifestyle. Therefore, it is a healthcare evolution where the new advances in genetic and molecular diagnosis made it possible to identify, based on the analysis of the tumour genotype and phenotype, the most effective, appropriate, and targeted treatment for distinct subpopulations of people with the same condition, which means, shifting from a "one-size-fits-all" to a "precision medicine" strategy (4,5,7,8).

II - Review of literature

1. Cancer: a leading cause of death

Although more recent lines of research undoubtedly show the success that has been seen in the fight against this disease, due to technology and science advances, the truth is that cancer is feared by all, given the high mortality rate associated with it on a global scale.

Cancer is not a novel disease. Around the year 400 BC, Hippocrates, the father of medicine, proposed the Humoral Theory of Medicine, which asserts that the body is made of four fluids or humours: blood, phlegm, yellow bile, and black bile. Any imbalance of these fluids, according to this theory, caused disease, and Hippocrates attributed cancer to an excess of black bile. He coined the terms "carcinos" and "carcinoma" to characterize cancers, being the first to use them. The word "cancer" comes from the Greek word "karkinos," which means "crab," and is thought to refer to the appearance of blood vessels in tumours that look like crab claws. Furthermore, cancer is not a uniquely human disease - all multicellular life, including plants and animals, are impacted (9,10).

Oncology is the medical speciality in charge with research and treatment of cancer. The prefix “onco” derives from the Greek word “onkos”, which means mass or nodule. It is also worth noting that cancer is linked to medical terms like neoplasm, which refers to the body's ability to generate new tissue - this characteristic distinguishes neoplastic diseases from other that require the destruction of cellular tissue, such as inflammatory/infectious and degenerative diseases (11).

This disease is a huge human health problem that affects anyone, regardless of age, gender, or socioeconomic status. It has become so common, that most likely we will face it at some point in our lives, whether we get it ourselves or know someone who does.

Cancer is defined as a complex group of genetic diseases that can affect any organ of the body, caused by an anomaly in the basic and fundamental unit of life - the cell. There are over 100 different forms of cancer, which are typically named by their location and tissue of origin (9,12,13).

Humans are complex beings with trillions of cells, with 330 billion of them being replaced every day due to the cell cycle. Cells contain genes that encode for proteins, which are responsible for nearly every task of cellular life. Under normal circumstances, the body

maintains a system of control and balance over cell proliferation, ensuring that cells divide only when new cells are needed. Furthermore, cells work together to conduct all the basic activities required for life (14,15).

Cancer cells, on the other hand, put this idea to test. These cells behave discordantly due to gene expression changes, that results in a disruption of the aforementioned system of control, and the balance of cell growth and cooperation between cells. Therefore, since the cell cycle correction procedures are incapable to function, there is an accumulation of genetic damage in cells over time, as such, they begin to divide and proliferate uncontrollably, ignoring regular replication orders in favour of pursuing their own reproductive ambitions, and eventually form a mass known as a tumour. These cells can also migrate through the circulatory and lymphatic systems, penetrating neighbouring tissues and forming aggregates in different parts of the body – metastasis -, ending in the individual's death. So, the hallmarks of cancer cells are their uncontrolled proliferation, invasion, and metastasis, as well as their ability to prevent cell death and immune system surveillance (2,14,16,17).

The cells' anomalies are caused by mutations in the genes, as previously mentioned, which can be hereditary, generated by errors in DNA replication, or triggered by chemical exposure that causes DNA damage (12).

The tumour can be either benign, when cells remain in their original location, or malignant, which, in opposition, is associated with the cells ability to grow in surrounding tissues and spread throughout the body via the lymphatic or circulatory system (18).

The Classification of Malignant Tumours is a widely used system for determining the precise stage of cancer, with each type of cancer having its own TNM classification. The prognosis of the disease, as well as treatment options, can be assessed by using this classification. TNM's features are categorized as follows: the T category denotes the primary tumour site and size; the N category represents regional lymph node involvement; and the M category indicates the presence or absence of distant metastatic dissemination (19).

1.1 Statistics

Cancer is a prominent cause of death and a significant obstacle to extending life expectancy in the whole world. In 2020, 1 in every 6 deaths was due to cancer, equating to near 10 million deaths. Estimates suggest that by 2040, this number will have risen to roughly 16 million fatalities (2,20).

In terms of incidence, around 19 million new cases were reported in 2020. This number is expected to increase about 56% by 2040, presenting a major threat to human life (20).

Breast, lung, colorectal, prostate, and stomach cancer were the 5 types of cancer with the highest incidence in all genders and all ages, according to GLOBOCAN 2020. Lung cancer causes more deaths each year than colon, breast, and prostate cancer combined. Moreover, with an estimated of 1.8 million fatalities, lung cancer remained the dominant cause of cancer death in 2020, followed by colorectal, liver, and stomach cancers (3).

A global pandemic was declared on March 11, 2020, which caused a dramatic impact on public health, mainly due to the fact that COVID-19 burdened the healthcare systems, causing a considerable reduction in the ability to treat non-COVID diseases, and cancer was not left out. On one hand, there was limited access to care due to the closure of health institutions and decrease in healthcare staff, such as doctors and nurses, due to infections and quarantine. On the other hand, individuals were afraid of getting infected and were hesitant to seek medical advice. Diagnoses were delayed and patients began to present with advanced stage cancer because of this circumstance, resulting in less effective therapy and thus higher mortality (21,22).

The global burden of cancer incidence and death is growing by leaps and bounds, not only due to population growth and aging, but also due to increased exposure to cancer risk factors that are related to socioeconomic development. Cancer has a major economic impact over the world, contributing to both healthcare expenses and lost productivity due to disease (morbidity) and premature death. Healthcare costs in the European Union totalled €57.3 billion, with morbidity and premature death causing productivity losses of €10.6 billion and €47.9 billion, respectively. The amount spent on oncological medications in Serviço Nacional de Saúde (SNS) Hospitals in Portugal has increased significantly in the last four years, from €285 million to €428.6 million. Since many types of cancers have a good chance of cure if diagnosed early and treated properly, early diagnosis, as well as proper therapy and care for cancer patients, can help reduce cancer burden and its costs (23–26).

1.2 Carcinogenesis

All types of cancers are caused by mutations in the DNA sequence, which can range from minor alterations, impacting only a few nucleotides, to extremely significant changes affecting the structure of chromosomes (27).

Although the DNA replication process is incredibly efficient, it has its flaws. As a result, mutations can develop naturally when cells divide. The repair of these mutations, which prevent the mutant cells from replicating, is essential for normalcy. For this to happen, cells have mechanisms for repairing and correcting DNA damage, as previously stated. However, the repair process is never 100% efficient, and DNA damage that is not repaired, leads DNA polymerase to malfunction, resulting in base pair sequence aberrations and mutation development (28).

Age, environment, food, and hereditary are features that have a role in the development of this disease. In terms of percentage, viruses are thought to be responsible for 5% of all human malignancies, whereas radiation accounts with 5% and chemicals with the remaining 90%. Focusing on the contribution of chemicals, tobacco use is believed to be responsible for 30% of these, while chemicals related with diet, lifestyle, and the environment are responsible for the rest (29).

The term "carcinogenesis" refers to the process of cancer development. This is a multi-step procedure that takes many years to be completed, with the 3 main steps being the initiation, promotion, and proliferation.

Phase 1 – Initiation

This step occurs when crucial cancer regulatory genes in adult stem cells are randomly mutated.

Phase II - Promotion

The mutant stem cell that emerged from the first event then endures throughout multiple cell division cycles and gives rise to a clone of mutant cells in this second step. Because of their mutagenesis, cancer cells proliferate continuously, providing them with advantages over normal cells in terms of growth and survival.

Phase III – Progression

In the third and final step, the clone spreads, and the daughter cells randomly undergo more mutations, allowing the cancer cells to acquire new features, such as infiltrating and destroying normal tissues, moving to distant locations of the body, and surviving. This procedure continues throughout the neoplasm's existence. The exceptional molecular heterogeneity that characterizes most malignant neoplasms is attributable to the various mutations that are acquired unequally by distinct subclones. (28)

Another important topic to discuss is tumour dormancy. Tumour dormancy is a period during which the disease is undetectable before it manifests itself as proliferative. This concept arose from clinical observations that cancer recurrence happens several years, if not decades, following surgical removal of the main tumour (30,31).

Tumour mass dormancy can be achieved by 2 pathways: angiogenic dormancy and immune-mediated dormancy. In the first, there is a balance between cancer cell proliferation and death, whereas in the second, disseminated dormant tumour cells evade immune surveillance and escape the immune system (30).

There is also an emerging type of tumour dormancy that is called cellular dormancy, which is characterized by minimum proliferation and minimum death. The cells are in a quiescent state (the cell cycle is stopped at the G0 phase). This state is required for cancer cells to acquire new mutations, survive in new environments, and initiate metastasis, besides developing resistance to cancer therapy, and avoiding immune destruction (30,31).

As it is known, antiproliferative medications primarily target rapid growing cells, as such, dormant cells can easily escape these therapies. This could be an issue as cancer cells dormancy can cause resistance to medicines (30,31).

1.3 Traditional cancer therapies

Nowadays, most types of tumours are treated with generalized classical therapies, which assume that all individuals with a certain type of cancer respond to a certain drug in the same way. Thus, depending on the type of cancer, all patients receive the same first-line therapy, although it may have very low effectiveness (4).

The three primary approaches of traditional cancer treatment are surgery, radiotherapy (local therapy) and chemotherapy (systemic therapy). However, since they are "one-size-fits-all" approaches, they lack in specificity, and so, have some drawbacks, such as harming non-cancerous tissues. As a result, many efforts are being made, mostly in the field of precision medicine, to identify novel treatment approaches (6,7).

According to statistics, the effectiveness of traditional cancer therapy is still an unresolved issue that is getting more prominent, indicating that the conventional treatment does not satisfy today's society needs or expectations (32).

1.3.1 Surgery

In conventional medicine, surgery is the primary treatment for most cancer cases. Moreover, it can be performed in different stages of the disease and with different goals, the main being: curative, palliative, or preventive (prophylactic) (32,33).

Nonetheless, most surgical indications correspond to curative surgery. Curative surgery is commonly used as the initial approach for primary solid tumours (localized in a specific and accessible area of the body) since it is assumed that in the early stages of the disease, as there are no organ metastases, this procedure may provide the best results, allowing for long remissions. This procedure entails the resection of the tumour or organ in which it is found, as well as the excision of the locoregional lymph nodes, often being conducted after earlier treatments with chemotherapy and/or radiotherapy to maximize the probability of total resection. Unfortunately, in nearly 60% of the cases, the disease is detected in an advanced stage, making surgical treatment ineffective (32–34).

Although this sort of surgery is intended to be curative, paradoxically, it can augment the spread of metastases, since it is linked to risk factors, including anaesthetic difficulties, infections, cancer cell dissemination in the bloodstream and immune system suppression, whereas the last two, are crucial for metastasis (32).

Palliative surgery is used at advanced stages. Even though it may not cure cancer, palliative surgery can be combined with other treatments to address an issue that is causing pain or incapacity. Some tumours in the abdomen, for example, can grow to the point where they cause intestine obstruction, requiring surgery to remove it. This type of surgery can also be used to treat pain that is not responding to medicines (33,34).

Preventive surgery is performed in people who have high risk of developing tumours in the future, even though, at the time of surgery, the tissue does not have cancerous cells. Hence, the purpose of this type of surgery is to lower cancer risk and aid in its prevention. For example, some women with a significant family history of breast cancer, have a mutation on BRCA1 or BRCA2 genes, and since the risk of breast cancer is substantial, prophylactic mastectomy may be considered. Another example is the polyps in the colon that may be considered as precancerous tissue, and surgery to remove them may be performed as a preventive measure (33,34).

1.3.2 Radiotherapy

Radiotherapy is described as the careful application of ionizing radiation on cancer cells, causing DNA damage and, as a result, the death or shrinkage of cancer cells. There are two major forms of ionizing radiation: photon radiation (X-rays and γ rays) and particle radiation (electrons, protons, neutrons, carbon ions, α particles, and β particles). It can be used alone or in combination with surgery, chemotherapy, or both, to improve therapeutic outcomes. The goal is to conduct radiation therapy in such a way that the quantity of radiation delivered to selected normal tissues is avoided or at least minimized, while the dose of radiation delivered to the tumour is maximized (35–37).

Radiation therapy can be split into two categories based on how the radiation is delivered: external radiation therapy and internal radiation therapy (38).

In external radiation therapy, the radiation source is placed at a certain distance from the patient, whereas for internal radiation, it is set near the tumour (inside the body). Moreover, the radiation source for internal radiation can be either solid - termed as brachytherapy -, or liquid. One example of brachytherapy is the use of iodine-125 seeds implanted in the prostate. The iodine-125 seeds are implanted transperineally to get a homogenous dose across the prostate volume. This procedure is used to treat patients with prostate cancer (35,38).

1.3.3 Chemotherapy

Chemotherapy is one of the most common treatments for cancer patients and is classified as a systemic treatment in the sense that the used drugs spread through the body. The purpose of this therapy is to cause as much harm to cancer cells as possible, while causing the least damage to healthy cells. That is the goal, but finding the right balance has not always been easy (39,40).

Cancer cells are known to have certain traits that, in theory, make them more sensitive to chemotherapeutic agents than normal cells, such as its rate of proliferation - which is faster than in healthy cells - and its difficulty to repair errors. However, most chemotherapeutic agents act non-specifically, harming both malignant and normal cells, particularly fast-growing cells namely, gastrointestinal, hair follicles, and immune system cells. This explains the most common side effects of chemotherapy known as nausea, hair loss and increased susceptibility to infections (33,41).

But why does chemotherapy fail? Tumour cells have a great ability to divide and have a high mutation rate, easily developing resistance. In terms of cancer cells species evolution, if a set

of cells, previously unable to metastasize, develops a mutation that leads them to produce a protein that permits metastasis, the reasoning is to go elsewhere. That is an advantage. There are several mechanisms associated with tumour resistance development, such as, increased drug efflux, improved repair/increased tolerance to DNA damage, high anti-apoptotic potential, lower permeability, and enzymatic deactivation are all mechanisms that enable cancer cells to survive chemotherapy.

Furthermore, there are two types of resistance: primary, when the tumour is not sensitive to the selected treatment, and secondary, when the tumour becomes resistant to a treatment which originally caused a response (42).

Multidrug resistance in cancer is caused by two different types of cell surface carrier proteins: the adenosine triphosphate binding cassette superfamily of transporters, that is an efflux pump that expels harmful chemotherapeutic medicines from cancer cells, and the solute carrier transporter superfamily, which regulates cellular uptake of anticancer medicines. Thus, the decrease of these carriers' activity can lead to drug resistance (43).

The immune system, in addition to treatment (drugs), should act as a brake to prevent these mechanisms from happening. However, even though tumour cells express antigens, the immune system may not recognize them since these cells create mechanisms to evade host immunity. Some examples include the downregulation of cancer-associated antigens' expression, in addition to the secretion of anti-inflammatory cytokines that overcome the activation of effector immune cells. Furthermore, in 2011, Allison and Honjo discovered immune checkpoints that block the recognition of tumours by immune cells, namely CTLA4 and PD-1/PD-L1, respectively. (44–46)

1.4 Lung Cancer

In 1912, Adler published the first monograph about malignant lung tumours. At that time, this type of cancer was considered one of the rarest in the world, with just 374 cases reported in the literature. Over the years, this disease has had a massive progression from being an extremely rare disease to becoming one of the cancer types with the highest incidence, and the leading cause of cancer-related death in both gender (3,47).

Lung cancer is defined as a solid tumour that develops from bronchial epithelial cells with several genetic mutations accumulated, resulting in a wide range of phenotypes. There are two main histological groups that respond differently to therapy: small cell lung cancer (SCLC) and

non-small cell lung cancer (NSCLC). Although SCLC has the worst outcome, with rapid tumour growth and metastasis in the early course of the disease, the most common is NSCLC, which accounts for 85% of all primary lung cancers. The SCLC contributes to the remaining 15% of the cases. Regarding the NSCLC, World Health Organization (WHO) subdivides it into three categories: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. (48,49)

Smoking is the major risk factor for lung cancer, accounting for about 80% of cases. Other risk factors are exposure to radon gas and occupational exposure to some compounds such as asbestos, arsenic, and benzene (environmental respiratory carcinogens). The risk of developing lung cancer is also enhanced by genetic factors and history of other lung disorders (e.g., chronic obstructive pulmonary disease, tuberculosis, and asthma) (50–52).

Despite decades of research, treatment options remain limited in terms of cure or significant survival benefit. Overall, the 5-year survival rate for lung cancer is 21%, whereas the NSCLC subtype being 26%, compared to 7% for SCLC. Unfortunately, only 24% of lung cancers are detected at an early stage, with a 60% 5-year survival rate. This highlights the need for better diagnosis and therapeutic options, including the identification of new therapeutic targets as well as predictive markers of treatment response (53).

1.4.1 Non-Small Cell Lung Cancer

Considering a histological classification, NSCLC is subdivided into squamous and non-squamous types. Within the latter, adenocarcinoma type accounts for most cases, while the large cell type represents a smaller percentage. Therefore, as aforementioned, we can say that NSCLC is subdivided into 3 main categories: squamous cell carcinoma, adenocarcinoma and large cell carcinoma.

I. Squamous cell carcinoma: it accounts for 25-30% of the cases, and is, more than any other type of NSCLC, strongly linked to smoking habits. It usually occurs in the central region of the lung or in the main airways, such as the bronchi, starting in squamous cell precursors (flat cells that line the inside of the airways in the lungs) (54).

   Squamous carcinomas' genetic profiling did not reveal any often, druggable mutations, hence, when it comes to targeted therapy, this tumour type is rarely mentioned (55).

II. Non-squamous cell carcinoma:

   a. Adenocarcinoma: is the most frequent type of NSCLC, accounting for roughly 40% of lung cancer. This type of lung cancer usually causes peripheral lesions and develops from

alveolar cells in the smaller airway epithelium, being identified as a malignant epithelial tumour with glandular differentiation, that can produce mucin and tends to exhibit immunohistochemical markers, such as napsin A or thyroid transcription factor 1 (TTF1). It is more common in smokers or ex-smokers, although it is also the most common type of lung cancer found in non-smokers. Beyond that, it is the most common type of lung cancer in younger people, and women are more affected than men (33,56,57).

b. Large cell carcinoma: it accounts for about 5% to 10% of all lung malignancies. This type of cancer can appear in any part of the lung, and it tends to spread and grow quickly, making treatment challenging. Since it lacks morphological traits that separate it from other histological subtypes, it is classified as an undifferentiated NSCLC. The incidence of large cell carcinoma is declining because of new immunophenotyping techniques that allow for a better classification of less differentiated squamous cell carcinomas and adenocarcinomas (56,58).

1.4.1.1 Diagnosis

Patients with lung cancer do not always show clinical symptoms in the early stages of the disease. This aspect, combined with inadequate screening programs, culminates in a diagnosis at an advanced stage, often in metastatic stage IV, when symptoms, such as cough, fatigue, dyspnoea, chest pain, weight loss and haemoptysis begin to develop. Late diagnosis translates into poor prognosis and lower patient survival. In this regard, clinicians must be more willing to evaluate high-risk patients, even if they have non-specific symptoms. It is also critical to enhance a screening approach for NSCLC diagnosis since the disease's clinical progression is correlated to the stage at the time of diagnosis (56,59–61).

There are several tools that a specialist can use to determine the tumour's stage and subtype, such as diagnostic imaging, biopsy by pathological evaluation and molecular tests. After identifying lesions through imaging techniques, a biopsy is required to confirm the diagnosis. The tumour's TNM stage must also be assessed. As a complement, there is also the information obtained through DNA sequencing of tumour tissue (solid biopsy). When tumour tissue samples are inadequate because the DNA extracted is insufficient and/or damaged, it is possible to use liquid biopsy as an alternative method, which tests for circulating tumour DNA (ctDNA), micro-RNA, and circulating tumour cells. This information can be crucial in determining the tumour's evolution and prognosis, as well as guiding treatment since it helps oncologists to better understand their patient's tumour heterogeneity (60,61).

2. Precision medicine: a new approach to cancer therapy

The terms "precision medicine" and "personalised medicine" are frequently used interchangeably in the scientific community. This occurs as a result of the National Research Council's definition of "personalised medicine," be quite similar to the definition of "precision medicine". Precision medicine studies a variety of factors affecting a person's condition, such as diseases, the environment, and lifestyle, to make it possible to identify distinct subpopulations of people with the same condition, whereas personalised medicine primarily focuses on medical treatments for a single person. In this monography, we will use the term "precision medicine" (62).

Precision medicine is a revolutionary breakthrough that questions current clinical practice standards and reshapes how cancer is classified and treated, becoming a new bet for improving medical services (63).

This approach highlights the role of molecular modifications as a cause of disease onset, considering an individual's genetic diversity, environment, and lifestyle to better predict whether an individual is predisposed to get a specific disease, to provide early diagnosis and treatment response, and to identify the most appropriate therapy. Therefore, precision medicine integrates data currently used for diagnosis and treatment (signs, symptoms, personal/family history, and commonly used complementary tests) together with an individual's genetic profile (7).

Precision medicine, combined with Next Generation Sequencing (NGS) technology, has allowed oncologists to sequence patients' genetic material, and take use of medications that target the genes directly involved in the cause of the disease, slowing its progression (64).

2.1 Precision Medicine: Myth or Reality?

It is well recognized that the traditional cancer treatment strategy is excessively simplified and is not designed to address the disparities among cancer diagnosis, resulting in inefficient and costly treatments, and unwanted side effects for patients. According to studies, 75% of cancer drugs used in chemotherapy have a limited effect, meaning that only 1/4 of patients benefit from treatment. As a result, genomic and genetic testing within the realm of precision medicine may play a role in the solution. In fact, they have been used in the last two decades, following the publication of the first version of the human genome project (8,65,66).

Recent scientific developments, particularly in the field of biotechnology, have enabled the identification of specific and complex biological traits connected to carcinogenesis, allowing cancer therapy to be tailored to the needs of individual patients (66).

Precision medicine advances promise to revolutionize the way we address diseases like cancer; nevertheless, not all patients will have access to novel diagnostics and therapies. As a result, it is becoming increasingly important for clinically valid tests to be implemented quickly in clinical settings. Precision medicine treatments are, in fact, more expensive than traditional therapies, however, these treatments reduce the need for additional testing and allows for the prescription of more effective therapies as a first-line treatment, improving patient outcomes and resulting in lower costs (67).

Several governmental projects are underway to realize the promise of precision medicine in oncology, and lots of precision medicine trials have been conducted. In recent years, breakthroughs in tumour biology and the understanding of the interactions between the tumour and the immune system have resulted in a significant progress in this field. However, it is the dramatic decrease in the cost of genomic testing that has pushed precision medicine to the forefront (65,66,68).

The initial steps in the genetic integration domain of cancer patients' treatment and guidance circuit are currently being carried out in Portugal. The IPO Porto has created its own Precision Medicine strategy and has enrolled over 330 patients in this ground-breaking program. The process is still very focused on identifying patients in the context of a clinical trial and is intended for patients who have exhausted all traditional therapy choices, allowing them to obtain treatment based on gene sequencing analysis and tumour mutation detection (tumour biomarkers), conceding access to novel medications in clinical trials or off-label (69).

Furthermore, the ''All of Us'' program, launched in the USA in 2015, is part of a new paradigm in healthcare. In this program, scientists, healthcare professionals, and technology specialists collaborate to produce individualized care that goes well beyond cancer treatment. This initiative seeks to enrol more than a million patients and use data from genomic sequencing to health care records over the course of ten years (70,71).

Overall, a total of 215 million dollars was allocated to various health-related projects. One of the large sums, $70 million, was allocated to the National Cancer Institute, which focuses on cancer research. The purpose was to extend genetic-based cancer clinical trials and investigate

fundamental aspects of cancer biology in order to produce more customized cancer medicines (72).

“16 of the 46 new molecular entities FDA approved in 2017 — as well as three gene therapies — are personalized medicines”, as we can see Precision Medicine is becoming progressively a reality (73).

Belgium, Norway, Estonia, France, and Israel all have programs in place as well (65).

2.2 Non-Small Cell-Lung Cancer in the Era of Precision Medicine

Even though histologic classification of lung cancer in SCLC and NSCLC is still widely employed, there has been a paradigm shift, owing to extraordinary advances in the genetics field. Molecular testing was introduced, becoming helpful in clinical practise to identify relevant genetic alterations for both diagnostic and therapeutic purposes, allowing for a more precise pathological and genetic classification of lung cancer. Cancer's molecular classification shows that there are several distinct diseases that should be recognized as distinct entities. Therefore, SCLC and NSCLC are thus no longer effective diagnostic alternatives for appropriately guiding therapy in the era of targeted molecular therapies - pathologists must now distinguish between cell types and, in addition, run a sophisticated battery of molecular testing, so that patients fully benefit from molecularly targeted drugs (74,75).

2.2.1 Diagnosis

One of the applications of precision medicine in cancer is the diagnosis. The goal is to use patient-specific data to characterize diseases to detect them early and, ideally, prevent them (76).

In the last years, it has been noticed that there are tumours that share characteristics (have altered genes in common) regardless of the organ in which they originated. In the case of lung cancer, molecular characterization has expanded our knowledge about cellular origins and molecular pathways affected in each of the sub-types. In NSCLC type, the discovery of driver mutations has enabled the use of medications that target these alterations, resulting in enhanced therapy efficacy and lower toxicity (8,74,77).

In the precision medicine field, methods for screening NSCLC patients for driver mutations and other abnormalities (molecular testing) have become increasingly essential to choose the correct treatment approach. These methods are in constant expansion, lacking standard platforms for testing (55,77).

Molecular testing can be performed in a range of methods, including hotspot testing for specific genes, or more complete tumour gene sequencing. Comprehensive NSCLC testing for many prognostic markers demands the examination of various biological molecules (DNA, RNA, and proteins), as well as the use of various analytical platforms (PCR, DNA sequencing, immunohistochemistry, and fluorescence *in-situ* hybridization (FISH). However, since many patients with advanced NSCLC do not have enough biopsy tissue to complete all the hotspot or gene sequencing tests, liquid biopsy or peripheral blood-based genetic testing have demonstrated to be helpful in detecting these gene mutations (55,78).

2.2.1.2 Fluorescence *in-situ* hybridization (FISH)

FISH is a cytogenetic-molecular approach that was first developed in the 1980s. This laboratory method is the gold-standard for identifying and locating specific DNA sequences on chromosomes, being used to determine DNA rearrangements in tumours. Therefore, it is a helpful tool in precision medicine, since it can detect a wide range of mutations, including translocations, insertions or inversions, deletions, and amplifications. In addition, FISH is frequently employed to validate gene-level amplifications, when immunohistochemistry (IHC) data are ambiguous (79,80).

FISH involves attaching an individual's full chromosomal set to a glass plate and then exposing it to a "probe", which is a small amount of pure DNA marked with a fluorescent dye. The fluorescent probe binds to segments of the chromosome with a high degree of sequence complementarity. The chromosome and sub-chromosomal site where the fluorescent probe binds can be observed using a fluorescence microscope, and therefore, the mutations can be easily detected (79,81).

Importantly, the Vysis Dual Color break-apart FISH was the first (and still the most widely used) FDA-approved detection method for Anaplastic Lymphoma Kinase (ALK)-positive NSCLC, in addition of being used to validate other ALK detection methods. Although it is a very sensitive method for identifying ALK locus disruptions, it cannot discriminate between the various ALK fusion partners. Moreover, FISH is an expensive technique that requires specialised knowledge to interpret the data and takes a long time to complete (82,83).

2.2.1.3 Immunohistochemistry (IHC)

IHC is a widely used method in many medical research laboratories for tissue-based diagnostics and biomarker identification. It can identify changes in proteins caused by gene aberrations

(most typically gene amplifications), as well as specific DNA rearrangements or point mutations (such as EML4-ALK translocation in NSCLC) but does not allow identification of the fusion partner. Furthermore, IHC has also been applied to biomarkers detection, namely PD-L1, which assesses if a patient is eligible for anti-PD-1/PD-L1 treatment (80,84).

The reasoning behind using IHC to diagnose NSCLC is, for example, that normal lung tissue does not exhibit detectable levels of ALK, but NSCLC ALK-positive expresses ALK at decent levels (55).

IHC is a less expensive technique than FISH, that requires less experience, and produces faster results. In addition, it is more widely available in hospital settings. This method is based on the assessment of protein expression using antigen-specific antibodies (80).

The heart of IHC process is the detection of the epitope of the antigen by an antibody. The antigen is usually a protein or glycoprotein. Furthermore, IHC assays used in clinical practice most commonly involve the use of a species-specific secondary antibody, besides the primary antibody. The primary antibody may be monoclonal or polyclonal, usually of the IgG class, and is generated by immunizing a mammalian host, such as mouse or rabbit. So, since it is generated against a specific epitope, it is destined to recognize it. In addition, the secondary antibody recognizes the Fc region of the primary antibody as an antigen and bind to it. Frequently, the secondary antibody is linked with a fluorophore, so that allows a fluorescence-based detection, with the use of a fluorescence microscope (85).

2.2.1.1 Next-Generation Sequencing (NGS)

In order to effectively manage NSCLC, a comprehensive molecular profiling is essential. This follows the growing importance of molecular markers in therapeutic outcomes. The answer to this need is the NGS technology.

NGS is a massively parallel sequencing technique used for analysing sequences of DNA and gene expression (RNA species). It was introduced between 2004 and 2006, and dramatically increased the output of sequencing data, revolutionising biomedical research. It is now one of the methodologies used in clinical practise and has recently been advised by the European Society for Medical Oncology (ESMO) to conduct a comprehensive molecular characterization in cancer patients (86–88).

When compared to the Sanger method employed in the early 2000s, which could only generate a few dozens of thousands of sequence data, NGS overcome its sequencing limitation, as it

made it possible to sequence hundreds to thousands of genes or gene regions simultaneously, while ensuring excellent accuracy and sensitivity due to its high sequencing coverage (89).

The underlying principles of Sanger and NGS technologies are identical. In both, DNA polymerase sequentially adds fluorescent nucleotides to a lengthening DNA template strand. By means of its fluorescent tag, each integrated nucleotide may be recognised. Nevertheless, the main difference between Sanger sequencing and NGS is sequencing volume (89).

Data shows that NGS technique is reliable and useful either for research and in the clinic as it improves diagnosis, prognosis, and treatment of cancer. Additionally, it is the most accessible method for describing genetic modifications in cancer patients using tumour tissue samples as well as circulating cell-free DNA. With the help of this technology, it is possible to identify each tumour's features and potential drug targets. In fact, this method has been applied in lung cancer to identify biomarkers for early diagnosis, to decide on a specific course of treatment, and to identify causal mutations. As such, NGS acts as a tool to assess some of the key gene mutations that contribute to the development of lung cancer, including Epidermal Growth Factor Receptor (EGFR), V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF), Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS), Human Epidermal Growth Factor Receptor 2 (HER2), ROS Proto-Oncogene 1 Receptor Tyrosine Kinase (ROS1), ALK, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA), Neurotrophic Tyrosine Receptor Kinase (NTRK), Rearranged During Transfection (RET), and Mesenchymal Epithelial Transition Factor Receptor (MET) (90,91).

Due to its significant advantages over FISH and IHC, which can have variable results depending on the pathologist's expertise and cannot be used on other types of samples, the NGS technique has emerged as the go-to method for analysing various sample types, and different subtypes of lung cancer (90).

NGS is already employed in many technologically equipped hospitals for NSCLC diagnosis and, in the medium to long term, it is anticipated to replace traditional gene analysis methods. However, despite its clear benefits, NGS is not yet readily available to all cancer centres around the world since it requires expensive, high-tech equipment and reagents, as well as powerful bioinformatic tools and specialised personnel, for both experimental and data assessment (55).

2.2.2 Therapy - Biomarkers and therapeutic targets

In the first decade of the 21st century, a significant advance in NSCLC management was made, revolutionizing the paradigm of detection and treatment of this type of cancer. This occurred as a result of a greater understanding of the molecular structures and signalling pathways of Receptors Tyrosine Kinase (RTK), which led to the development of a number of targeted therapies, including tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (MAbs). There are currently available TKI that target the most successful targets in lung cancer, such as the EGFR, and the vascular endothelial growth factor receptor (VEGFR). The latter, when agonized by its natural agonist, stimulate the creation of new vessels (angiogenesis), which is an important step for the tumour's ability to isolate itself from the outer world - in terms of drug access -, as well as to increase its access to nutrients (77,78,92).

In the field of precision medicine, biomarkers are essential for diagnosing a disease, identifying different subpopulations of patients who are most likely to benefit from a specific treatment, and monitoring the patient's response to that treatment. The most useful biomarkers for assessing the success of targeted therapy in advanced NSCLC are somatic genome mutations known as "driver mutations" (77).

The research of ALK, ROS1, RET, and NTRK translocations, as well as the detection of EGFR, BRAF, KRAS and MET mutations, are already part of the diagnostic standards for NSCLC, and inhibitors of these kinases are currently employed in clinical practise. In addition, for mutation/translocation-negative tumours, PD-L1 IHC studies may be required to aid in the selection of immune treatment. (55)

In this section, we will review the most relevant biomarkers and therapeutic targets in the management of NSCLC.

2.2.2.1. Epidermal Growth Factor Receptor (EGFR)

The EGFR, commonly referred to as HER1 or ErbB-1, is a transmembrane TKR that is the prototype of the ErbB family. Erb-B2 (HER-2/Neu), Erb-B3 (HER-3), and Erb-B4 (HER-4) are the other three members of this family. The ErbB family has been shown to have a significant role in the initiation and maintenance of a range of solid tumours. Furthermore, the first RTK suspected of activating oncogenic signalling was EGFR. This receptor is commonly upregulated in breast, colon, head-and-neck, renal, ovarian, and NSCLC (93–95).

Epidermal growth factor (EGF) is a common mitogenic factor that promotes the proliferation of various cell types, mainly fibroblasts and epithelial cells. The EGFR is activated by the EGF, and this in turn starts intracellular signalling (96).

Normal cells are thought to have between 40000-100000 EGFRs, whereas in cancer there is an overexpression of more than 10^6 EGFRs per cell. In normal epithelial cells, EGFR mediates numerous essential processes, including proliferation, survival, differentiation, adhesion, and migration. However, these receptors are dysregulated in cancer, and its mutations over-activate pro-oncogenic signalling pathways, such as the Phosphoinositide 3-Kinase - Protein Kinase B - Mammalian Target of Rapamycin (PI3K/AKT/mTOR) and RAS/RAF/MEK/ERK [also called Mitogen-Activated Protein Kinase/ Extracellular signal-Regulated Kinase (MAPK/ERK)], which lead to uncontrolled cell proliferation, enhanced cell angiogenesis, and increased cell invasiveness, contributing to cancer spread and growth, being one of the most well-studied cells signalling pathway (74,93).

Overall, the discovery of EGFR activating mutations, the development of EGFR TKIs, as well as the exceptional clinical success of these drugs in EGFR mutant lung cancer patients, laid the groundwork for lung cancer precision medicine (95).

In the United States, mutations in the EGFR TK are found in around 15% of NSCLC adenocarcinoma, and they are three times more common in non-smokers than smokers, and in women than men. Furthermore, the rate of EGFR mutations is significantly higher among Asian people. The typical strategy to decide whether or not to use an EGFR TKI for the initial therapy of a patient with advanced NSCLC is to look for the presence or absence of a driver mutation in EGFR (55,97).

In terms of structure, EGFR is made of an extracellular ligand-binding domain, a hydrophobic transmembrane domain, a cytoplasmic TK domain, and a C-terminal domain. Since this receptor is found on the cell surface as a monomer, it must dimerize to activate the TK. Thus, the signalling cascade starts when the ligand (EGF) binds to EGFR, inducing its homodimerization or heterodimerization, and transphosphorylation of various tyrosine residues on the C-terminal tail. The phosphorylated tyrosine residues provide a docking site for proteins containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, leading to a complete signalling network associated with a variety of outcomes, including cell proliferation, growth, differentiation, migration, and apoptosis inhibition, as aforementioned (74,93,95).

Essentially, the goal of the EGFR TKI is to block the activation of downstream signalling induced by EGFR and cause tumour cells to die.

EGFR mutations can arise in any domain, however transmembrane mutations are unusual. The kinase domain of the EGFR is affected by two major types of mutations that were identified in exons 19 and 21. They are detectable by all diagnostic tools and account for 85–95% of druggable EGFR alterations. Exon 19 contains an in-frame deletion (Del746-750), which accounts for most EGFR-activating mutations. The L858R substitution found in exon 21, is a point mutation (CTG to CGG), that results in the replacement of leucine by arginine at codon 858, being responsible for around a third of the cases (55,74,94,95).

The last two mentioned mutations make tumours very responsive to EGFR TKIs, therefore, first (Gefitinib and Erlotinib) and second generation (Afatinib) EGFR TKI have been approved as standard first-line therapy in advanced EGFR positive NSCLC. In the case of NSCLCs with the Del746-750 mutation, first, second, as well as third generation (Osimertinib) EGFR TKIs, show greater responses when compared to those carrying the L858R mutation (55,95).

Erlotinib and Gefitinib are reversable first-generation EGFR TKIs that inhibit downstream signalling and prevent tyrosine phosphorylation by competing with endogenous ATP for the kinase domain binding. Erlotinib was approved by the FDA in 2004 as a third-line treatment for advanced NSCLC, and later in 2013, as first-line treatment for EGFR mutant NSCLC. Gefitinib initial approval dates 2003, and later, in 2015, it was approved as first line treatment of metastatic NSCLC with EGFR mutations (94,98).

Afatinib (approved in 2013) is a second-generation EGFR TKI that forms an irreversible chemical covalent bond with the receptor by binding irreversibly to the free cysteine in the kinase domain. Gefitinib is usually the best tolerated drug in the first-line setting, even though Afatinib is slightly more effective (94).

Since the occurrence of an EGFR mutation in advanced NSCLC confers a better prognosis and highly predicts sensitivity to EGFR TKIs, targeted therapy should be the first option, even before chemotherapy and immunotherapy (77,97).

The EGFR inhibitors discussed above can often reduce tumours for months or even years, but for most patients, these medications eventually stop functioning, owing to secondary mutations in the EGFR gene in cancer cells. In fact, these uncommon somatic mutations are found in about 10% of EGFR-mutated cancers, and although they also appear to be susceptible to EGFR

TKIs, they seem to be less responsive than the ex19del and L858R mutants. The list of rare EGFR mutations includes exon 18 nucleotide alterations, exon 19 in-frame insertions, exon 20 alterations and exon 21 mutation L861Q. In the case of exon 20, an important mutation is the T790M, that results from the substitution of methionine for threonine at position 790. This is the most frequent acquired mutation associated with resistance to pharmacological EGFR TKIs, causing resistance to both Gefitinib and Erlotinib (55,93,99,100).

Although the mechanism of T790M resistance is uncertain, the longer methionine side chain may operate as a steric barrier to TKI binding, causing lung tumours to become resistant to Erlotinib or Gefitinib and resume measurable growth within 6 months to 2 years, in most patients. The third-generation inhibitor - Osimertinib - is recommended for the treatment of EGFR T790M-mutated tumours since it has a unique action against this receptor isoform. Furthermore, this drug is approved as a first-line NSCLC therapy because it prevents the T790M substitution from occurring early and prolongs progression-free survival (55,74).

Unavoidably, cancer cells will undergo further mutations, rendering resistant to third generation EGFR-TKIs, resulting in disease progression. As a result, a complete understanding of how resistance occurs, as well as how to design effective ways to delay or overcome resistance, is a key challenge and a pressing requirement.

2.2.2.2 Anaplastic lymphoma kinase (ALK)

ALK is a transmembrane RTK that belongs to the insulin receptor superfamily. Although the physiological role of ALK is not fully understood, evidence suggests that it plays a regulatory role in the normal development and function of the central and peripheral nervous systems. Furthermore, this TK is expressed improperly in a variety of tumours, with 3 to 5% of NSCLC, predominantly the adenocarcinoma subtype, exhibiting chromosomal rearrangements involving the ALK gene loci on chromosome 2 (101,102).

For instance, the oncogenic EML4-ALK fusion gene is a crucial target for NSCLC, as it is restricted to NSCLC patients that do not have EGFR nor KRAS mutations (103).

Moreover, patients with tumours carrying ALK fusion oncogenes or variants are often younger and light or non-smokers (102).

The ALK was first discovered in anaplastic large-cell lymphoma (ALCL) as a fusion gene called nucleophosmin (NPM)-ALK fusion. It is characterised by a translocation between chromosomes 2 and 5, which leads to constitutive activation of ALK and downstream signalling

pathways that promote oncogenesis. Furthermore, it was the first mutation in the ALK gene discovered in human cancers (104).

Since the identification of the NPM-ALK fusion gene in ALCL, several ALK fusion partners have been discovered in other cancers. The most typical ALK rearrangement in NSCLC is the fusion oncogene EML4-ALK. It forms from an overlap of the 5' end of the echinoderm microtubule-associated protein-like 4 (EML4) gene with the 3' end of the ALK gene. (101,102)

Importantly, four signalling pathways have been identified as carcinogenic mediators of ALK deregulated activity: Janus kinase - signal transducer and activator of transcription (JAK-STAT), RAS/RAF/MEK/ERK, Phospholipase C (PLC), and PI3K/AKT/mTOR. All these pathways are recognised regulators of cell cycle progression, proliferation, and apoptosis; thus, a deregulation of these pathways is a typical hallmark of malignant tumours (102).

Furthermore, testing for this fusion gene in NSCLC is significant since "ALK-positive" cancers respond well to ALK TKIs.

Over the last 10 years a lot of work has gone into developing ALK-targeted medicines. In 2011, FDA approved the first medicine for ALK-positive NSCLC – Crizotinib. However, patients quickly developed resistance to the treatment, requiring the development of more effective drugs (101).

Then came the second generation ALK inhibitors such as Ceritinib and Alectinib. Ceritinib is approximately 20 times more potent than Crizotinib, and in 2017, FDA granted approval of this drug for patients TKI-naïve and Crizotinib-resistant with metastatic NSCLC ALK-positive (102,105).

Furthermore, Alectinib was approved in 2017 as first-line treatment of patients with metastatic NSCLC ALK-positive. This drug showed benefit of longer-term follow-up of clinical trials when compared to others second generation ALK inhibitors (101).

The third generation ALK inhibitor – Lorlatinib – was approved by the FDA in 2021 for patients with metastatic NSCLC ALK-positive in the front-line setting and may be considered another option for first-line treatment. Moreover, this drug proven efficacy in patients with ALK-rearranged lung cancer previously treated with first- and second-generation ALK inhibitors (101).

According to current guidelines, in patients with newly diagnosed, ALK-positive NSCLC, it is recommended a next-generation ALK inhibitor as first-line treatment. Brigatinib is a next-

generation ALK inhibitor that received FDA approval in May 2020 as a first-line treatment option for patients with advanced (stage IV) NSCLC. This drug targets a broad range of ALK mutations and has demonstrated improved efficacy over Crizotinib (101).

2.2.2.3 Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS)

Nowadays, there is an exponential development of plenty therapies with promising outcomes, for several molecular targets that once were thought to be "undruggable". KRAS is a good example. Attempts to target KRAS had been unsuccessful until recently, when covalent inhibitors that target the KRAS G12C substitution were approved after showing positive outcomes in clinical trials. KRAS belongs to the RAS proteins family, a family of plasma membrane anchored guanosine triphosphate hydrolyses (GTPases), that has been widely studied in humans. Other than KRAS, the RAS proteins family has two other members extensively studied, namely NRAS and HRAS, but in RAS mutant cancer patients, KRAS is the most frequently mutated isoform. Moreover, KRAS mutation is the most typical genetic abnormality found in human cancer, with a prevalence of around 30%. In Western countries, KRAS mutations account for 20–25 % of lung adenocarcinomas, while in Asian countries, they account for 10–15 % (106,107).

KRAS plays an essential role in the controlled activation of many signalling pathways involved in cell proliferation, differentiation, cytoskeleton dynamics, vesicle trafficking, secretion, and protein translocation to membranes (108).

Furthermore, this protein has two different activity states: when bound to GTP, it will be activated, transducing the activating signals to downstream pathways, such as the MAPK/ ERK pathway and the PI3K/AKT/mTOR signalling pathway; whereas when bound to GDP, it will be inactivated. As such, when KRAS is mutated, it remains perpetually activated, causing uncontrolled cell proliferation (106).

KRAS mutations are mostly found in hotspots (codons 12, 13, 59, and 61), with codon 12 accounting for nearly all of them (95%). The most common KRAS-mutant form in non-smokers is glycine 12 to aspartic acid (G12D). Moreover, glycine 12 to valine (G12V) is also commonly found in this group of patients. Glycine 12 to cysteine (G12C), on the other hand, is the most common mutation among current and previous smokers (55).

KRAS G12C is the most common mutant isoform, accounting for 41–49% of NSCLC cases with G12 mutations. In fact, this mutation has generated a lot of interest as it is accessible to allosteric inhibition (55,109).

So far, Sotorasib and Adagrasib, are the two KRAS G12C-specific inhibitors, that showed good clinical outcomes in patients with advanced stage KRAS G12C-mutant solid tumours, previously treated (106).

Sotorasib, acts as a specific inhibitor of the KRAS-G12 C variant by selectively binding to the P2 pocket of the switch II domain of this isoform, generating an irreversible inhibition. This medicine received accelerated FDA's approval on May 28, 2021, for adult patients with KRAS G12C mutant locally progressed or metastatic NSCLC who have had at least one prior systemic treatment. Moreover, this medicine was authorized by the European Medicines Agency (EMA) in the beginning of 2022 (106,110).

Adagrasib is also a molecule that directly and irreversibly inhibits KRAS-G12C. It has been designated as a breakthrough therapy by the FDA, based on positive single-agent activity in patients with NSCLC who had a KRAS G12C mutation. In addition, a marketing authorization application for Adagrasib has been submitted to the EMA (106,111,112).

Other medicines have been developed in addition to these two, such as JNJ-74699157 and BI-1701963. The first is a molecule targeted against the P2 pocket in the switch-II region of KRAS-G12C, that enrolled in clinical trials despite minimal preclinical evidence being reported. However, its safety profile was deemed insufficient for future clinical development. The second molecule, BI-1701963 is referred as a pan-KRAS inhibitor as it does not inhibit the protein itself, but it does inhibit the guanine nucleotide exchange factor (GEF) protein SOS1, which is among the most important GEF associated with RAS proteins. By inhibiting SOS1, KRAS remains in the inactivated state linked to GDP, and the signalling cascade cannot be initiated. Furthermore, BI-1701963 was the first GEF-KRAS inhibitor to enter clinical trials (106,113).

2.2.2.4 V-Raf murine sarcoma viral oncogene homolog B (BRAF)

BRAF is a protein kinase that regulates cell signalling, growth, and survival. This protein is normally inhibited by negative feedback, however, when BRAF mutations occur, the RAS/RAF/MEK/ERK pathway remains activated, resulting in uncontrolled cell growth and proliferation (114).

BRAF mutations are uncommon in NSCLC, accounting for only 2% of all lung adenocarcinomas. Furthermore, they are more common among never-smokers, women, and aggressive histological types. Moreover, in standard clinical practice, they are categorised as V600 mutations or non-V600 mutations, the second ones being found in around 50% of all BRAF mutations in NSCLC. The BRAF V600E mutation in exon 15 is the most common activating BRAF mutation found in solid tumours other than lung cancer. This point mutation corresponds to a valine to glutamate substitution at codon 600 (114,115).

Sorafenib was developed as a targeted therapy against BRAF mutant kinase. However, according to a study, the antitumor activity of Sorafenib is connected with EGFR mutation status other than KRAS mutation status, which led to an unclear understanding of whether Sorafenib could be used as a BRAF inhibitor or not (114,116).

Novel-generation BRAF inhibitors Dabrafenib and Vemurafenib are ATP-competitive inhibitors of BRAF kinase. Both are highly specific for BRAFV 600E mutations. Furthermore, Vemurafenib was found to be ineffective in patients with BRAF non-V600 mutants (77,114).

Although monotherapy appeared to be an efficient therapeutic option at first, with high response rates, further research has revealed that a combination of BRAF and MEK inhibitors is the best approach. In that regard, FDA has approved Dabrafenib, and Trametinib - a type of MEK inhibitor - as a combination regimen focusing on BRAF pathway inhibition (77).

2.2.2.5 ROS proto-oncogene 1, receptor tyrosine kinase (ROS1)

ROS proto-oncogene 1 is a member of the insulin receptor family which also encodes for a receptor TK (105).

When ROS1 is mutated, the TK enters in a state of constitutive activation, which promotes cancer cell proliferation. This mutation is rarely found in NSCLC, accounting for only 1-2% of cases, being predominantly associated to the histological subtype adenocarcinoma. Additionally, it affects more frequently younger people, women, and people with no significant smoking history (74,105).

Several ALK-targeting drugs, including Crizotinib, Ceritinib, and Lorlatinib, have been reused as ROS1 inhibitors due to its genetic and clinical similarities with the ALK-positive NSCLC. Furthermore, Entrectinib inhibits both ROS1 and Neurotrophic tyrosine receptor kinase (NTRK). The NTRK gene family contains three members, NTRK1, NTRK2 and NTRK3, which produce Tropomyosin Receptor Kinase (TRK) proteins, namely TRKA, TRKB and

TRKC. These proteins regulate cell proliferation, differentiation, apoptosis, and survival of neurons in both the central and peripheral nervous systems, playing key roles in nervous system development. Furthermore, in addiction to nervous system, TRK receptors are found in a variety of non-neuronal cell types and tissues, such as monocytes, lung, bone, and pancreatic beta cells. When there are rearrangements in NTRK genes, it can result in two genes fusing together, and consequently, producing altered TRK proteins, which can lead to uncontrolled growth of cancer cells (105,117).

It is interesting to note that even though ROS1 and ALK rearrangements have clinical similarities and homology, it is unusual to find both mutations at the same time. Additionally, it is uncommon to see ROS1 mutations along with EGFR and KRAS mutations simultaneously (105).

2.2.2.6 TP53 (Tumour-Suppressor Gene Tumour Protein p53)

The p53 protein is a tumour-suppressing protein present in cells throughout the body, encoded by the TP53 gene. This protein, p53, is known as the "guardian of the genome", due to its essential role in controlling the cell cycle, apoptosis, autophagy, and DNA repair in response to harmful substances (118,119).

A wide variety of malignancies that are challenging to treat are linked to p53 dysfunction. It can occur from TP53 gene mutations that result in defective, misfolded proteins, or from an excess of a negative regulator of the p53 tumour suppressor, the mouse double minute 2 (MDM2) protein (118).

It is known that more than 50% of cancers show changes in p53 signalling, making TP53 the most frequently mutated gene in humans. Additionally, TP53 mutations are present in about 50-60% of NSCLC, with the majority being missense mutations. When TP53 is mutated, it ceases to operate as a tumour suppressing gene, and acquires features that promote cancer progression (119).

According to epidemiological research, TP53 mutations are strongly connected with smoking habits. Furthermore, a growing body of research suggests that TP53 mutations are linked to a worsening prognosis and cancer drug resistance (120).

One of the most frequently mutated sites of human TP53 is codon 273. It has been demonstrated that the mutant TP53-273H, which most frequently has the substitution arginine to histidine, possesses both dominant-negative and gain-of-function characteristics. TP53-273H still has

partial sequence-specific DNA-binding and transcriptional activation capabilities, unlike the majority of tumour-derived mutant TP53 proteins. Therefore, it is possible that TP53-273H mutations might result in increased cell proliferation, abnormal DNA recombination, increased genomic instability, and decreased chemotherapeutic effectiveness (119).

The prevalence of TP53 mutations is a crucial predictor and biomarker for patients' prognosis and response to targeted therapies. There are still no licenced medications that can specifically target the p53 pathway in cancer, despite an impressive body of research regarding the function of p53 and its numerous mutations. However, there are some interesting lines of approach under study, such as compounds that counteract the disruptive effects of p53 mutations or neutralize the excessive p53 suppression caused by the MDM2, seen in some tumours. Additional developments in these and other therapeutic modalities, along with the discovery of carefully considered combination medicines, may finally result in a decisive triumph over a variety of cancer types (118).

2.2.2.7 Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA)

It is known that the PI3K/AKT/mTOR signalling pathway plays an important role in tumorigenesis and cancer development, since it is involved in a wide range of cellular and biological activities, including cell proliferation, metastasis, apoptosis, metabolism, and cell cycle regulation. This pathway is typically dysregulated in lung cancer as genetic changes affecting one of its constituents may cause PI3K activation and malignant transformation (121).

PI3Ks are heterodimeric lipid kinases that can be divided into three classes (I - III) according to their structure and biochemical properties. Human cancer is strongly associated with class IA PI3K that consists in a catalytic subunit (p110) and a regulatory subunit (p85). The regulatory subunits p85α, p85β, and p85γ are encoded by PIK3R1, PIK3R2, and PIK3R3 genes, whereas the catalytic subunit p110α, p110β, and p110δ are produced by PIK3CA, PIK3CB, and PIK3CD, respectively. Furthermore, activating mutations in the PIK3CA gene have typically been found in two crucial sites: exons 9 and 20 that encode the helical and catalytic domain of p110α, respectively (122).

The oncogene PIK3CA plays important roles in somatic cell survival, differentiation, and proliferation, among other crucial physiological functions. It is expressed in healthy breast, brain, lung, and other tissues, usually in an inactive form. However, mutations in PIK3CA may

lead to its overexpression, which can subsequently activate the PI3K/AKT/mTOR pathway and promote cell carcinogenesis by abnormally enhancing the catalytic activity of PI3K (121).

Interestingly, although PIK3CA gene is one of the most commonly mutated gene in cancer, such somatic mutations are relatively uncommon in lung cancer and only occur in about 5% of NSCLC cell lines (122).

Moreover, increased chromosomal copy number (through amplification or polysomy) is another form of oncogene activation in addition to mutations, and it is known that lung tumours typically amplify the region of chromosome 3q (3q25-27), where PIK3CA (3q26) is located (123).

Although there is significant room for improvement on the development of tools to target the PI3K signalling pathway and the downstream mediators, some encouraging results have already been reported. In fact, research using the PIK3CA inhibitor Alpelisib (the first PI3K inhibitor for breast cancer approved by the FDA) has showed greater survival than that of other treatments, suggesting that it may also be effective for lung cancer (90,121,122).

In addition to the medicines that target the biomarkers discussed in this section, there are several ongoing clinical trials on NSCLC, with examples provided in Table 1.

Table 1 - Examples of ongoing clinical trials on NSCLC

Study Title	NCT Number	Phase	Interventions
Clinical Trial of YH25448(Lazertinib) as the First-line Treatment in Patients with EGFR Mutation Positive Locally Advanced or Metastatic NSCLC (LASER301)	NCT04248829	3	Drug: Lazertinib 240 mg/160 mg Drug: Gefitinib 250 mg Drug: Lazertinib-matching placebo 240 mg/160 mg Drug: Gefitinib-matching placebo 250 mg
First-in-Human Study of JNJ-74699157 in Participants with Tumors Harboring the KRAS G12C Mutation	NCT04006301	1	Drug: JNJ-74699157
Adagrasib in Combination with BI 1701963 in Patients with Cancer (KRYSTAL 14)	NCT04975256	1	Drug: MRTX849 Drug: BI 1701963
A Study of BPI-7711 Capsule in Non-small Cell Lung Cancer Patients	NCT03866499	3	Drug: BPI-7711 Drug: Gefitinib Drug: Placebo Tablet Drug: Placebo capsule
A Study of XZP-5955 Tablets in Patients with NTRK or ROS1 Fusion Positive Locally Advanced or Metastatic Solid Tumours	NCT04996121	2	Drug: XZP-5955 tablets

IV – Conclusion and Future Perspectives

Lung cancer was classified by a wide histologic classification, but currently is a complex disease, comprising many molecular subtypes with diverse prognostic and therapeutic implications.

It is known that the traditional cancer treatment strategies such as surgery, radiotherapy, and chemotherapy, are not designed to address the disparities among cancer diagnosis, resulting in inefficient and costly treatments, and unwanted side effects for patients, not being able to provide a comfortable lifestyle. As such, the development of high-throughput diagnostic techniques and bioinformatics tools allowed for the identification and characterization of genomic biomarkers, as well as for the acquisition of deeper knowledge on cancer biology, ushering a new age in precision oncology therapy. One remarkable example is the next-generation sequencing, that provide quick and high-volume testing of genome, allowing the analysis of multiple targets. By providing a more comprehensive tumour profile for each patient, this technique enables physicians to select therapies based on precise diagnoses and tumour characteristics. Consequently, it can enhance prognosis, therapy choices and outcomes, while protecting patients from treatments that might not provide the desired clinical benefit.

The assessment of actionable mutations has become a standard in the management of this disease since it was found that mutation-targeted treatments boost patients' quality of life and extend their survival.

Additionally, it is important to recognise that non-squamous NSCLC has largely been responsible for the advancements in precision medicine, since it carries a lot of druggable mutations, while squamous NSCLC did not reveal any often.

The EGFR was the first RTK suspected of activating oncogenic signalling, laying the groundwork of precision medicine in the field of oncology. Gefitinib and Erlotinib, first-generation EGFR inhibitors, have revolutionised the way EGFR mutant NSCLC is treated and represent the potential of precision medicine. However, their efficacy has been constrained by the emergence of resistant clones, which inevitably cause disease progression. The second-generation inhibitor – Afatinib – has a promising potential in patients with EGFR mutant NSCLC, since it showed to be slightly more effective than Gefetinib. More recently, third-generation EGFR inhibitor – Osimertinib – has demonstrated potential in overcoming resistance induced by the acquired T790M mutation.

Since ALK and ROS1 are closely related kinases, TKIs, such as Crizotinib, Ceritinib, Lorlatinib have been applied in both ALK- and ROS1-rearranged cancers. Furthermore, Alectinib and Brigatinib have been approved for the treatment of ALK-mutant NSCLC, while Etrectinib can be employed for the treatment of tumours with ROS1 rearrangement.

When it comes to KRAS, attempts to target this gene had failed until recently, when covalent inhibitors targeting the KRAS G12C substitution – Sotorasib and Adagrasib – were approved following positive clinical trials results. Furthermore, BI-1701963 is a GEF-KRAS inhibitor that is currently in clinical development.

For BRAF-mutated NSCLC, current guidelines recommend a combination of BRAF and MEK inhibitors – Dabrafenib plus Trametinib –, respectively, as a standard targeted therapy option.

It is well recognized that the p53 dysfunction is a major driver of cancer development mainly because, in the absence of this "guardian of the genome," cells are no longer properly protected from mutations. Unfortunately, despite lots of research there are no approved drugs that specifically target the p53 pathway in cancer.

PIK3CA mutations are among the most prevalent gene alterations seen in human cancers, however their prevalence in NSCLC is low. Even though there have not yet been many positive results reported for inhibitors of PIK3CA-mutated NSCLC, Alpelisib, a breast cancer inhibitor, may be effective in treating this type of cancer, as it has shown greater survival than other therapies.

Future perspectives suggest that NSCLC multigene testing will most likely become more standardised during the next ten years. NGS seems to be the method that can meet all needs, but to fully replace the current diagnostic techniques, it needs to be more cost-effective and user-friendly.

Furthermore, despite significant improvements in research and understanding of the molecular processes underlying lung cancer progression, there are still a variety of challenges to overcome, such as:

(1) the requirement to find unidentified driver gene mutations in the group of individuals for whom there are no current targeted medicines;

(2) a deeper comprehension of the mechanisms underlying resistance as well as a sensitive and specific methods to assess it;

(3) the development of novel treatment regimens that can target several biological pathways essential for the survival and spread of lung cancer.

In essence, collaboration between the medical and scientific communities is essential to ensuring that precision oncology has the right opportunity to widely revolutionize the treatment of most cancer patients.

V – References

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