In the era of personalized medicine, the continuous increase in the number of treatment response biomarkers has significantly altered the clinical management strategies for patients with non-small cell lung cancer (NSCLC). In this context, the epidermal growth factor receptor (EGFR), as per the guidelines issued by international societies such as IASLC/AMP/CAP, has served as the gateway for molecular characterization in NSCLC patients.

Following the clinical benefits observed in patients harbouring EGFR molecular alterations, clinical studies have expanded the spectrum of biomarkers approved for clinical practice by integrating additional molecular alterations. These range from the detection of point mutations in genes such as BRAF and KRAS to the evaluation of clinically relevant gene rearrangements in ALK, ROS1, RET, and NTRK. An additional layer of complexity in molecular characterization aimed at detecting alterations predictive of sensitivity to targeted therapies is the inclusion of mutations leading to aberrant exon 14 skipping in the MET gene within the panel of "must-test genes." In this landscape, key challenges remain in harmonizing pre-analytical procedures for handling biological material intended for genomic profiling, implementing molecular characterization technologies capable of

addressing oncologists' clinical questions, and generating a comprehensive yet user-friendly report for the oncologist managing the patient. These aspects continue to be the subject of discussion and debate to optimize the diagnostic and clinical workflow for NSCLC patients.

In recent years, we have witnessed a revolution in the approval of targeted therapies for clinical practice in patients with NSCLC. The rapid and progressive expansion of therapeutic strategies available for oncologic patients derives from the increasing number of biomarkers approved by international societies such as CAP, IAP, and IASLC, for which clinical studies have demonstrated a clinical benefit from targeted therapy administration. In this context, it is essential to distinguish a minimal panel that includes biomarkers already approved for clinical practice (EGFR hotspot mutations, KRAS p.G12C, BRAF mutations; aberrant rearrangements in ALK, ROS1, RET, NTRK; MET exon 14 skipping mutations, and PD-L1 receptor expression) from biomarkers approaching approval based on clinical trial indications (ERBB2).

Historically, single-test technologies real-time polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) have been widely used in pathology laboratories for genomic profiling; however, they are now inadequate due to their limited reference range and low applicability in detecting emerging biomarkers. A major challenge in the molecular characterization of aberrant rearrangements lies in the technical limitations of conventional methodologies (e.g., Fluorescence in situ Hybridization (FISH) in detecting all fusion partners. Additionally, the high variability in fluorescence signal interpretation by operators further limits the clinical applicability of these techniques.

Due to these limitations, the transition to analogue-digital technologies such as next-generation sequencing (NGS)—which enables both qualitative and quantitative molecular alteration analysis in NSCLC patients eligible for targeted therapy—has become a logical consequence of the current diagnostic landscape. Moreover, NGS technologies are versatile in generating reliable molecular data regardless of the type of starting sample. Given these benefits, NGS systems have increasingly permeated clinical practice, leading to the development of platforms and assays with distinct technical

parameters. However, the technological heterogeneity of NGS platforms necessitates harmonization studies to optimize diagnostic workflows based on genomic analysis of real-world samples. This effort is critical for preparing molecular pathology laboratories to evaluate next-generation biomarkers (NRG1, BRCA1/2, MAP2K1), which are currently under investigation in phase III clinical trials for NSCLC patients. Considering the continuous expansion of molecular data available to oncologists, the integration of NGS platforms into clinical practice requires the development of a hierarchical network of multidisciplinary groups. These groups must manage molecular complexity and translate genomic findings into clinically actionable information for oncologic patients.

The current clinical landscape for patients with advanced NSCLC has greatly benefited from the molecular revolution, leading to the definition of new diagnostic classes that incorporate molecular profiling data alongside traditional morphological characterization. The application of NGS platforms has significantly increased the number of actionable alterations identified in NSCLC patients, revealing that approximately 70% of these cases harbour known molecular drivers. Building on these findings, the same approach has begun to unveil the molecular basis of small-cell lung cancer (SCLC), suggesting that this tumor subtype may also benefit from molecular profiling. In the advanced disease setting, NSCLC treatments are stratified into first- and second-line options based on molecular data obtained from genomic analysis. Among known biomarkers, EGFR has been a cornerstone of personalized therapy, with the clinical approval of tyrosine kinase inhibitors (TKIs) designed to selectively target cancer cells harbouring EGFR alterations. Advances in research have led to the development of new TKI classes with efficacy profiles tailored to specific EGFR mutations. Notably, Osimertinib, a third-generation TKI originally developed to target the p.T790M resistance mutation in EGFR exon 20 after progression on first-generation TKIs, has demonstrated high response rates as a first-line therapy in patients with EGFR-mutated NSCLC.

The need for molecular characterization is further highlighted by the use of ALK inhibitors, where treatment response is highly dependent on the specific rearrangement detected. Although ROS1 rearrangements are present in only ~2% of oncogene-addicted NSCLC cases, they are associated with

an overall survival of 51.4 months, with objective responses observed in all patients with this molecular marker.

The landscape of BRAF-targeted therapies has evolved with the combination of ERK and BRAF inhibitors, significantly improving objective response rates, progression-free survival, and median overall survival (OS) in patients harbouring BRAF codon 600 mutations. Similarly, despite their low prevalence in NSCLC, NTRK gene fusions render patients eligible for treatment with selective inhibitors targeting the chimeric protein.

RET rearrangements, often associated with specific immunophenotypic characteristics, have demonstrated substantial clinical benefit in terms of progression-free survival (PFS) and objective response rates (ORR) in patients with this molecular alteration. In the context of oncogene-addicted NSCLC, MET is considered a multiparametric biomarker. Currently, MET exon 14 skipping mutations qualify patients for specific inhibitor treatments, while additional therapeutic strategies are under investigation for copy number variations and gene rearrangements involving MET. Pending approval by the Italian Medicines Agency (AIFA) is the KRAS p.G12C mutation in exon 2, which has shifted the clinical paradigm for advanced NSCLC. The development of covalent inhibitors that selectively bind the receptor hosting this structural modification has demonstrated significant clinical benefit in the second-line setting.

For NSCLC patients without predictive molecular alterations, advanced-stage treatment relies on immune checkpoint inhibitors (ICIs) with or without chemotherapy as a first-line strategy, based on PD-L1 expression status. However, clinical studies have shown highly heterogeneous outcomes depending on the specific ICI used in clinical practice.

In the era of personalized medicine, the diagnostic classification of patients with advanced-stage lung cancer has been enriched through the integration of molecular data, enabling tailored clinical pathways for oncology patients. The close collaboration between oncology and pathology has led to the development of diagnostic workflows centred on assessing the molecular status of various biomarkers, which are now fundamental for clinical stratification. The pivotal role of pathology in

this setting derives from its responsibility in selecting the biological sample for molecular biomarker profiling. Moreover, PD-L1 receptor expression is an integral part of the biomarker panel currently available in clinical practice, further highlighting the essential role of pathology in this context.

The expansion of clinically relevant biomarkers in the coming years will lead to the identification of a new class of patients whose therapeutic strategies will be optimized based on the development of novel targeted pharmacological approaches. Among these biomarkers, EGFR remains the most extensively characterized in both biological and clinical contexts. Notably, a major research focus is dedicated to deciphering the molecular mechanisms underlying resistance to EGFR-targeting TKIs, including alterations in TP53, PIK3CA, and HER2. The identification of novel molecular targets associated with resistance to EGFR-directed therapies has paved the way for alternative treatment strategies, such as the use of next-generation drugs like Amivantamab, which specifically targets EGFR exon 20 insertions and has demonstrated superior clinical benefit compared to conventional chemotherapy.

Several research avenues are also exploring the potential of antibody-drug conjugates (ADCs) as an effective and well-tolerated therapeutic option in NSCLC. This strategy has shown promising results, particularly in patients with ALK rearrangements, improving ORR and PFS. The importance of accurately characterizing molecular alterations has led to further advancements in treatment selection for NSCLC, as evidenced by clinical trials such as UNICORN and ARTICUNO, which have emphasized differential clinical responses based on the specific molecular alteration detected. Exon 20 insertions remain an area of active investigation, with monotherapy strategies or ADC-based combination therapies targeting EGFR representing the latest frontiers in NSCLC treatment. Currently, the standard approach for BRAF-driven NSCLC involves combination therapy with MEK inhibitors, while new second-line treatment strategies are emerging, showing promising clinical performance in patients harbouring the KRAS p.G12C mutation. Within ALK-rearranged NSCLC, different resistance mechanisms to selective ALK inhibitors have been identified. In this setting, liquid biopsy plays a crucial role in detecting resistance mechanisms in patients who experience

radiological progression. To maximize clinical outcomes in patients progressing after ALK inhibitor treatment, novel therapeutic agents are under evaluation. Similarly, post-treatment resistance mechanisms in patients with ROS1 rearrangements are also under investigation, with early data on next-generation ROS1 inhibitors demonstrating substantial response rates and progression-free survival in both preclinical and clinical studies. Building on these innovations, targeted therapies for RET and NTRK rearrangements are also advancing, with new drug classes under development aimed at optimizing clinical pathways for NSCLC patients.

The number of predictive biomarkers for treatment response in the clinical stratification of patients with NSCLC is evolving, as the increasing number of biomarkers introduced into clinical practice adds complexity to laboratories performing molecular testing. Furthermore, the extreme heterogeneity in the approval of individual biomarkers across European countries further complicates the landscape. In this context, it is important to distinguish between real-life data, derived from clinical practice, and data from clinical trials. The development of databases like ATLAS allows for the systematization of technical, clinical, and molecular data, supporting the oncologist's ability to access and interpret complex molecular profiles that include co-mutations impacting a patient's clinical history. In this setting, harmonizing analytical procedures proves to be most effective when analyzing complex biomarkers like MET, which is expected to undergo a shift in role in the coming years, becoming a "triple" marker for patient selection in molecular-targeted treatments for NSCLC. Studies show that working with RNA becomes crucial when investigating molecular alterations such as aberrant translocations or mutations causing exon 14 skipping in the MET gene, helping to define the functionality of the identified molecular alteration. Given the exponential increase in biomarkers being introduced into clinical practice, tissue samples are inadequate for comprehensive molecular profiling in 40% of cases. Small tissue samples (biopsies or cytological preparations) show comparable adequacy to surgical resections in predicting therapeutic response in NSCLC patients. The use of massively parallel sequencing-based testing strategies in clinical practice allows for exhaustive molecular profiling, reducing overall costs compared to singleplex technologies when at

least four biomarkers are being tested. Despite these improvements to analytical protocols, a significant proportion of patients would not benefit from molecular-targeted therapies if integrative diagnostic tools (such as liquid biopsy) had not been introduced into clinical practice. Liquid biopsy encompasses a range of liquid biological sources from which various analytes (nucleic acids, circulating tumor cells, proteins) can be isolated, overcoming the analytical challenges of managing tissue samples, which still persist in clinical practice. In light of this innovation, it is important to consider both tissue and liquid biopsy as complementary aspects of the same diagnostic pathway, aimed at acquiring molecular data useful for clinical practice. Due to the intrinsic characteristics of liquid biopsy, new clinical management scenarios for oncological patients have revealed its potential applications, justifying its use in the early stages of the disease or in the detection of disease in healthy individuals.

To summarize, the pre-analytical phase, particularly in the management of tissue biopsies and peripheral blood samples, is a crucial step in the diagnostic process for NSCLC patients. The handling, processing, and preservation of samples before the analysis significantly influences the accuracy and reliability of molecular diagnostics. Effective management ensures high-quality results that guide clinical decisions. In NSCLC patients, tissue samples are often compromised in terms of the quality and quantity of nucleic acids extracted, which can hinder the analysis. The use of NGS platforms is beneficial, as they allow for a more comprehensive analysis of biomarkers that are relevant in clinical practice. NGS delivers critical molecular information that enables oncologists to obtain a more accurate picture of the molecular landscape of the tumor, enhancing the potential for personalized treatment strategies. The use of optimized and standardized bioinformatics pipelines for molecular data interpretation is essential for ensuring accurate results. Integrating first- and second-level analytical algorithms allows for better management of molecular data, especially when sample quality may affect the analysis. These bioinformatics tools help interpret complex molecular alterations and facilitate clinical decision-making, ensuring that results reflect the true molecular profile of the cancer. The harmonization of analytical procedures is achieved through ongoing

training of qualified personnel who handle biological samples from the pre-analytical phase through to molecular data interpretation. This continuous professional development ensures that best practices are maintained across all stages of sample management and analysis, leading to consistent, high-quality results. The moment of reporting, which is often carried out within a multidisciplinary team of experts, is the final stage of the analytical process. The final report must be clearly and concisely written, allowing clinicians to easily understand the molecular findings and incorporate them into the patient's treatment plan. The report should be actionable, providing guidance on therapeutic decisions based on the molecular profile of the tumor.

Finally, the integration of high-quality pre-analytical practices, advanced analytical technologies like NGS, bioinformatics pipelines for data interpretation, continuous staff training, and a collaborative multidisciplinary approach to reporting are all key elements that ensure a robust and effective diagnostic process for NSCLC patients. These steps contribute to providing accurate, actionable molecular information that can guide personalized treatment decisions and improve patient outcomes.

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