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The storm of NGS in NSCLC diagnostic-therapeutic pathway: How to sun the real clinical practice

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ABSTRACT

The increasing number of approved drugs along with next generation sequencing (NGS) technologies look out as potential revolution of biomolecular characterization of non-small-cell lung cancer (NSCLC). Nevertheless, several aspects impact on success rate of NGS in clinical practice: a multidisciplinary approach and thorough knowledge of strengths and limits of each technologic diagnostic tool are required. Crucial preliminary step is the selection of the best available sample before testing, aware of clinical condition and setting of disease. Genomic data should be than integrated in the clinical context and matched with available therapeutic options; Molecular Tumor Boards (MTB) are worldwide emerging interdisciplinary groups implemented to transfer the impact of precision medicine in clinical practice. In order to guarantee equity in treatment, these considerations should find their application widely and rapidly.

Aim of this review is offering an overview of emerging biomarkers, relative upcoming targeted drugs, and new diagnostic chances with an authors' perspective about a real-life diagnostic-therapeutic algorithm useful for daily clinical practice.

1. Introduction

Currently, alongside with the well-established predictive biomarkers, novel alterations are ingoing the European clinical practice and this highlights the need for a better defined testing strategy of non-small cell lung cancer (NSCLC) patients. There are several testing approaches that can be applied, depending on tumor alterations and technical capabilities, in order to allow a time-saving process.

Sample triage is essential for the correct management of these critical patients. Moreover, all considerations should take into account the possibility of a heterogeneous and decentralized local testing landscape, as in the Italian situation. The aim of this review is to give an overview on upcoming drug approvals and predictive markers in oncogene-addicted NSCLC patients, to discuss opportunities and challenges of next generation sequencing (NGS), including different platforms and panels, and finally to propose a diagnostic algorithm potentially useful

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within a Molecular Tumor Boards.

2. Upcoming drug approvals for oncogene-addicted NSCLC

It has been widely demonstrated that non-squamous lung carcinoma is not a single disease but a cluster of distinct molecular subtypes each defined by an oncogenic genetic variant. Gene mutations, rearrangements and amplifications have dramatically changed the treatment landscape of lung cancer since they have provided the rationale for targeted therapies.

So far, current national and international guidelines recommend testing for oncogenic targets (*EGFR*, *KRAS*, *ALK*, *ROS1*, *BRAF*, *RET*, *MET, NTRK* and *HER2*), along with immune-checkpoint inhibitor biomarkers (PD-L1) in order to select advanced stage NSCLC patients for currently approved targeted therapies (Mosele et al., 2020).

2.1. Mutations-targeting agents

2.1.1. The exon 20 insertions epidermal growth factor receptor (EGFR)

Since recommendation in 2011 by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) for all patients with lung adenocarcinoma, *EGFR* mutational assessment has become mandatory in the routine diagnostic practice of molecular predictive pathology laboratories (Beasley and Milton, 2011). In *EGFR*-mutant advanced NSCLC patients, first (gefitinib and erlotinib) (Mok et al., 2009; Rosell et al., 2012) ; second (afatinib and dacomitinib) (Yang et al., 2015; Wu et al., 2017) and third generation (osimertinib) (Soria et al., 2018) tyrosine kinase inhibitors (TKIs) targeting *EGFR* mutations are currently approved (Papini et al., 2021); of note osimertinib showed to be superior to the standard EGFR TKIs in the first-line setting in the FLAURA trial with an improvement in progression free survival (PFS) (18.9 months vs. 10.2 months) and overall survival (OS) (at 18 months 83 % vs. 71 months) respect to the other EGFR TKIs. In addition, it has been demonstrated the possibility to adopt a combination of first generation EGFR TKIs with chemotherapy or antiangiogenic therapies as a first-line treatment for patients with *EGFR*-mutated NSCLC patients with a significant improvement in clinical outcomes (Chen et al., 2021). Beyond *EGFR* deletions in exon 19 and p.L858R point mutation in exon 21, insertions within exon 20, detected in 1–2 % of metastatic NSCLC patients, as well as other "uncommon" mutations in exons 18, 20 and 21 (p.G719X, p.L861Q, p.S768I), need to be tested for an adequate treatment management (Ramalingam et al., 2020). Among these, careful attention should be paid to *EGFR* exon 20 insertions. These latter, identified in 1.5–3.0 % of NSCLC patients, are located in tyrosine kinase domain of EGFR protein (Gristina et al., 2020). As a general rule, these alterations can be grouped as in-frame insertions and three to 21 bp duplications within residues D761 and C775 (Vyse and Huang, 2019). Overall, *EGFR* exon 20 insertions lead to an inward rotation position of the αC-helix and the stable dimerization and constitutive activation of EGFR protein (Eck and Yun, 2010).

More recently, approvals and ongoing trials are targeting exon 20 insertions, with mobocertinib (TAK-788) receiving in April 2020 the Food and Drug Administration (FDA) breakthrough therapy designation for patients with *EGFR* exon 20 mutant NSCLC that have progressed following platinum-based chemotherapy (Takeda, 2021). Moreover, the ZENITH20−20 study is also evaluating safety and efficacy of poziotinib (HM781−36B) in patients with previously treated NSCLC and *EGFR* exon 20 insertions. The phase 2 study data show that the overall response rate (ORR) has been met as primary endpoint with durable responses (Socinski et al., 2020). These two compounds may be considered as a potential option for *EGFR* exon 20 mutant patients although the safety profile appears not easily manageable. Finally, amivantamab, a bispecific antibody targeting both EGFR and MET, received FDA accelerated approval for patients harboring *EGFR* exon 20 insertions previously treated NSCLC, based on the multicenter non-randomized study CHRYSALIS. This study showed an ORR of 40 %, with a median Duration of Response (DoR) of 11.1 months, with acceptable safety profile (Park et al., 2021).

2.1.2. The Kirsten rat sarcoma viral oncogene homolog (KRAS) G12C mutations

KRAS exon 2 p.G12C mutation are identified in about 12 %–14 % of NSCLC patients (Malapelle et al., 2021). KRAS is a G-protein with GTPase activity (Gimple and RAS, 2019). *KRAS* mutations determine the constitutive activation of KRAS protein and the subsequent signal transduction (Takács et al., 2020). *KRAS* mutations in NSCLC are more frequently reported in smokers (30 % vs. 10 %), and after years of failed efforts to target *KRAS* in lung cancer, positive results have been emerged from several recent clinical studies. The CodeBreaK 100 with the administration of sotorasib (AMG-510) showed a positive ORR for *KRAS* exon 2 p.G12C-mutant advanced NSCLC patients that failed a median of two treatments lines with chemo- and/or immunotherapy (FierceBiotech, 2021). A global Phase 3 clinical trial has recently reached the recruitment target of patients for comparing sotorasib to docetaxel in *KRAS* exon 2 p.G12C-mutant NSCLC patients (CodeBreaK 200 study). Additionally, a compassionate use program is currently open for sotorasib in pretreated patients. Another promising drug, adagrasib (MRTX849) has showed responses in 45 % of NSCLC patients from phase 1/1b and phase 2 clinical trials; but phase 2 is still enrolling to increase the target population and better assess drug safety. Some trials are evaluating combinatory approaches between the novel KRAS drugs and other inhibitors targeting different molecules (SHP-2 inhibitor TNO-155) with encouraging results in anti-tumor activity in some solid tumors in the pre-clinical phase. In September 2020, it has been announced the combination strategy of BI 1701963, a SOS1 pan-KRAS inhibitor, and adagrasib in solid tumors patients with *KRAS* exon 2 p.G12C mutation (Mirati, 2021).

2.1.3. The Mesenchymal-epithelial transition factor (MET)

c-*MET* receptor tyrosine kinase (*MET*) gene alterations have been reported in about 4.3 % of NSCLC patients (Cancer Genome Atlas Research Network, 2014a). These latter may lead to aberrant activation of downstream pathways (*RAS/ERK/MAPK, PI3K/AKT*, Wnt/beta-catenin, and *STAT*).

Among these, *MET* exon 14 skipping (*MET*ex14), that may occur mutually exclusive or in conjunction with *MET* amplification, has acquired a high relevance (Malapelle et al., 2020). *METex14* are characterized by the loss of exon 14 that determines a decreased degradation of the MET protein and increased activation of downstream signaling pathways (Yang et al., 2020; Drusbosky et al., 2021; Moosavi et al., 2021). Currently, testing for *MET*ex14 in advanced or metastatic disease, either for adenocarcinoma or squamous cell carcinomas is recommended by National Comprehensive Cancer Network (NCCN) guideline in order to administrate the FDA-approved drug capmatinib (Ettinger et al., 2021; Novartis, 2021). More recently, the GEOMETRY mono-1 trial evaluated highlighted the efficacy of capmatinib in advanced stage NSCLC harboring *MET*ex14 or *MET* amplifications.

Of note, limited efficacy has been observed in pretreated patients with *MET* amplifications and gene number copies less than 10, whereas a stronger increase in the ORR (from 7 to 12% to 40 %) in naïve patients has been reported (Wolf et al., 2020). Beyond capmatinib, another type Ib MET inhibitor, tepotinib, has received FDA accelerated approval for advanced NSCLC harboring *METex14* basing on data from phase II VISION clinical trial [https://www.fda.gov/drugs/resources-information-approved-drugs/fda-grants-accelerated-approval-tepotinib-metastatic-non-small-cell-lung-cancer].

In this trial, tepotinib has been administered in monotherapy in patients with advanced NSCLC *MET*ex14 assessed on liquid and/or tissue biopsies (Paik et al., 2020).

2.1.4. The human epidermal growth factor receptor 2 (HER2)

HER2 exon 20 insertions have a high grade of similarity with those in

EGFR and are related to the same two major structural regions, the αC-helix and the loop region (Friedlaender et al., 2021).

Three different alterations have been described for *HER2* in NSCLC: gene amplification, overexpression and *HER2* point mutations, accounting overall for about 5–7 % of naive lung alterations. Nevertheless, *HER2* mutations, particularly YVMA 776–779 insertions within exon 20, are emerging as a druggable target for targeted therapies in lung cancer and account for 80 %–90 % of all *HER2* mutations (Yoshizawa et al., 2014; Zhao and Xia, 2020).

In May 2020, as a result of the interim analysis of the Phase II global DESTINY-Lung01 clinical trial, trastuzumab deruxtecan received the FDA breakthrough designation for the treatment of patients with metastatic *HER2*-mutant NSCLC with disease progression on or after platinum-based therapy (Enhertu, 2021). The same trial failed to show effectiveness of the designed treatment in HER2 positive (IHC 3+ protein overexpressing or IHC 2+ with gene amplification) neoplasms, and clinically meaningful tumor response was only achieved in the mutant cohort. The mechanism of action of the drugs relies on the combination of trastuzumab, directed against *HER2*, with a cytotoxic payload called deruxtecan so that the compound is classified as Antibody Drug Conjugate. As the antibody seeks out and binds to HER2-expressing cancer cells, deruxtecan is then released to inhibit DNA replication, leading to cell death of the target and the immediately proximal cells (Smit et al., 2020).

Several trials have been set up in order to provide insight on the use of small HER2 TKI molecules in NSCLC. Some of these therapeutic agents have been used in *HER2* mutant or amplified patients, with controversial data and underline the great variability of clinical efficacy of HER2 therapeutic options in these NSCLC molecular subtypes (Kris et al., 2015; De Grève et al., 2015).

2.2. Gene fusions-targeting agents

2.2.1. The proto-oncogene tyrosine-protein kinase receptor (RET)

About 2% NSCLC patients harbor *RET* gene fusions leading to the production of abnormal RET proteins that can act as oncogenic drivers (Cancer Genome Atlas Research Network, 2014b). These gene rearrangements involves the carboxy terminal region of RET and various upstream gene partners, resulting in the constitutive activation of the fused protein (Drilon et al., 2018a). The initial treatment strategy developed for *RET*-rearranged NSCLC was based on a multi-targeted tyrosine kinase inhibitor (Drilon et al., 2016) while more recently novel next-generation selective RET inhibitors have been investigated and received the FDA approval for their highly selectivity.

The international, phase I/II LIBRETTO-001 trial led to the approval of selpercatinib in adult patients with metastatic *RET* fusion-positive NSCLC. Selpercatinib is a highly selective oral TKI with a profound anti-kinase activity against *RET* rearranged tumors that has shown a 64 % ORR in patients previously treated with at least a platinum-based chemotherapy and an 85 % ORR among untreated patients and highly effective also on intracranial metastasis (Drilon et al., 2020).

In the phase I/II ARROW trial, pralsetinib, another strong FDA-approved *RET* inhibitor has been found to have an ORR of 56 % in advanced *RET* fusion-positive NSCLC, irrespective of prior treatment or *RET* fusion types (Oxnard et al., 2018). Currently, the phase III randomized clinical trial AcceleRET is ongoing with the primary aim of comparing pralsetinib with the standard of care in first line treatment of metastatic NSCLC (NCT04222972).

2.2.2. Neurotrophic tyrosine kinase (NTRK)

NTRK1, *NTRK2* or *NTRK3* genes, which encode the neurotrophin receptors TRKA, TRKB and TRKC, have been described as undergoing fusion events leading to carcinogenesis in various adult and pediatric solid tumors. *NTRK* gene fusions can be found with different frequencies across several cancer types, with the challenge of their identification due to the higher incidence described in rare cancer, while a lower one is detected in the more frequent big killer type of tumors (Roviello et al., 2020). *NTRK* gene fusions were first identified in NSCLC in 2013 (Vaishnavi et al., 2013), and the frequency of these fusion events is low and specifically, *NTRK1* gene fusions are detected in about 3% of NSCLC cases while *NTRK2* and *NTRK3* in about 1% of cases, across all types (Rolfo and Raez, 2017).

The attempt of targeting these alterations across all tumor types has successfully led to the first approval of such agnostic therapeutic approach in Europe with the European Medicine Agency (EMA) licensing larotrectinib in September 2019 both in adult and pediatric metastatic or unresectable cancers (EMA, 2021). Efficacy was then confirmed based on pooled data from three major clinical trials: LOXO-TRK-14001, SCOUT, and NAVIGATE (Drilon et al., 2018b).

However, the identification of *NTRK* gene fusions for the clinical trials was prospectively determined in local laboratories using next generation sequencing or fluorescence in situ hybridization (Moosavi et al., 2021). ESMO recommendations on the standard methods to detect *NTRK* fusions in daily practice propose, for low recurrence unselected population, an immunohistochemical screening followed by RNA-sequencing for positive case (Marchiò et al., 2019). NGS panel for significant lung gene could be considered an upfront valid option.

The national drug agencies are indeed facing the new challenge of evaluating an agnostic drug (NICE, 2021; IQWIG, 2021).

The pivotal phase II STARTRK-2, phase I STARTRK-1 and phase I ALKA-372−001 trials, and data from the phase I/II STARTRK-NG study helped for EMA approval of entrectinib of adult and pediatric patients with *NTRK* fusion-positive solid tumors and for people with *ROS1*-positive advanced NSCLC. Tumor shrinkage happened in more than half of people with *NTRK* fusion-positive, locally advanced or metastatic solid tumors with an overall response rate of 63.5 % and an objective responses was observed across 13 tumor types (Rozlytrek, 2021).

3. Clinical algorithm: role of NGS, opportunity and pitfalls

3.1. Clinical power of the best choice, the magic triangle: sample, technique and panel

Tissue still represents the “gold standard” starting material for molecular analysis, including NGS. Nevertheless, tissue remain an issue in advanced stage NSCLC patients. In the vast majority of these patients the only available material for molecular analysis is represented by scant tissue samples (small histological biopsies and/or cytological specimens) (Aisner et al., 2016). Formalin fixed and paraffin embedded (FFPE) tissue samples (histological samples or cytological cell blocks) do not require an additional validation process before routine implementation for NGS analysis respect to cytological preparations (such as direct smears and liquid based cytology samples) (Lindeman et al., 2013). However, even these non-FFPE samples acquired a relevant role in the correct diagnostic molecular management of advanced stage NSCLC patients, as stated in the updated version of the molecular testing guideline from the CAP/IASLC/AMP (Lindeman et al., 2018). In fact, non-FFPE samples yield higher quality nucleic acids compared to FFPE specimens, which suffer from formalin fixation and lead to C > T artifacts that may determine false negative or false positive molecular results (Cree et al., 2014). Conversely, non-FFPE samples may be affected by the limited quantity of available material (Bellevicine et al., 2017).

Regarding molecular testing, different molecular assays are currently available, including NGS (Vigliar et al., 2015a). This latter enables, through a “sequencing by synthesis” approach, the analysis of different biomarkers for different patients, simultaneously (Vigliar et al., 2015b). As a general rule, NGS platforms can adopt one of the three following sequencing approaches: by synthesis, by hybridization, and by ligation (Reuter et al., 2015). Briefly, despite different platforms are currently commercially available, NGS workflows follow the same steps: (1) library preparation; (2) clonal amplification of single generated fragments; (3) massive parallel sequencing, and (4) data analysis (Vigliar

et al., 2015a). Regarding library preparation, Ion Torrent platforms (Thermo Fisher Scientific, Waltham, MA) employ a polymerase chain reaction (PCR) approach adopting multiple primer pairs that allow to select specific genomic targets (Rothberg et al., 2011), whereas the Illumina ones (Illumina, San Diego, CA) implement a hybridization-capture approach (Loman et al., 2012). The second step is represented by clonal amplification that allows the generation from a single fragment of hundreds of thousands of copies. This phase is obtained with an emulsion PCR on beads when considering the Ion Torrent platforms (Merriman et al., 2012), or with an emulsion PCR on a solid support on a flat glass microfluidic channel (flow cell) when adopting Illumina platforms (Mardis, 2013). The third phase is performed on solid chips able to identify the pH changes determined by the release of a hydrogen ion (H+) originated from the incorporation of non-labeled nucleotide by DNA polymerase by Ion Torrent platforms (Slatko et al., 2018). The Illumina platforms, instead, adopted labeled nucleotide to identify the incorporation by DNA-polymerase (Mardis, 2013). Finally, all generated data requires specific bioinformatics pipelines to be adopted for clinical purposes (Gargis et al., 2012).

Another crucial step for NGS analysis is the choice of gene panels able to satisfy diagnostic purposes. Currently different gene panels are commercially available (Hynes et al., 2017a). In this setting, there is the possibility to employ: narrow gene panels, covering up to 10–15 actionable genes; broad and clinically relevant panels, covering up to 50 genes, useful to enroll patients in clinical trials; tumor comprehensive panels, covering up to 150 cancer specific genes for translational research; human cancer comprehensive panels, covering up to 400 cancer relevant genes (Hynes et al., 2017b).

In the experience of an Italian molecular predictive laboratory, NGS workflow has been optimized to process tissue and liquid biopsy specimens for DNA- and RNA-based clinically relevant biomarkers by using two narrow, custom NGS panels (Malapelle et al., 2017; De Luca et al., 2021). In this setting, DNA-based approach is fundamental for point mutations, insertions and deletions detection, whereas may be limited when gene fusions are considered, due to the presence of intronic regions involved in the gene rearrangements. This limitation may be overcomes by a RNA-sequencing approach, however, pre-analytical issues, due to RNA less stability than DNA, may arise (Bruno and Fontanini, 2020).

In summary, as represented in Fig. 1, the most appropriate NGS result can be obtained combining the choice of the most suitable sample in terms of quality and quantity of DNA/RNA yield with a technical approach able to maximize the clinical sensitivity in the specific analytic context coupled to the most appropriate panel within the respective clinical context.

3.2. What, when and how: a proposed diagnostic algorithm

Despite the agreement on the importance of broad molecular testing approach for patients with lung cancer, there is still some difficulties in its full applicability in worldwide clinical practice. A recent international survey conducted by the International Association for the Study of Lung Cancer (IASLC) revealed that almost globally, the adoption of molecular testing for lung cancer is suboptimal with molecular testing rates of less than 50 % in more than 60 % of survey responders. Furthermore, most patients with molecular data provided, had only *EGFR* and *ALK* tested. The study also highlighted major barriers in testing cost, access, quality, turnaround time, and lack of awareness (Smeltzer et al., 2020).

In this context, pathologists and molecular biologists are central figures in what can be called the "*lung cancer sample triage*". The importance of a strict and continuous communication between all the professionals involved in the entire process, (pathologists, biologists and clinicians) is crucial. Indeed, the adoption of reflex biomarker testing required by pathologist at diagnosis, may potentially increase testing rate and shortening time to results (Anand et al., 2020). Since most pathology laboratories have been equipped in the last decade with platforms for single-gene analysis, technology update along with maintenance of equipment and expertise on orthogonal methods, could represent crucial aspects. Particularly, single-gene test strategy cannot be entirely considered outdated. It remains a valid option when rapid turnaround time is required or sample is not suitable to pass quality and quantity controls prior to NGS testing. In patients with deteriorating conditions the adoption of a rapid testing may be a rescue option and could be of crucial importance to save patient's life.

Whereas a broader genomic assessment should be always performed in NSCLC patients whenever possible, there is now a broad consensus about role of liquid biopsy as a complementary approach to tissue based analysis (Reita et al., 2021). Even if it may be not yet considered totally an alternative to tissue testing, molecular profiling on liquid biopsy in NSCLC is recommended at the time of initial diagnosis in patients with advanced NSCLC, when tumor tissue is scarce and/or unavailable, or finally for those patients in whom invasive procedures may be harmful (Rolfo et al., 2018; IASLC, 2021). These data are supported by the NILE

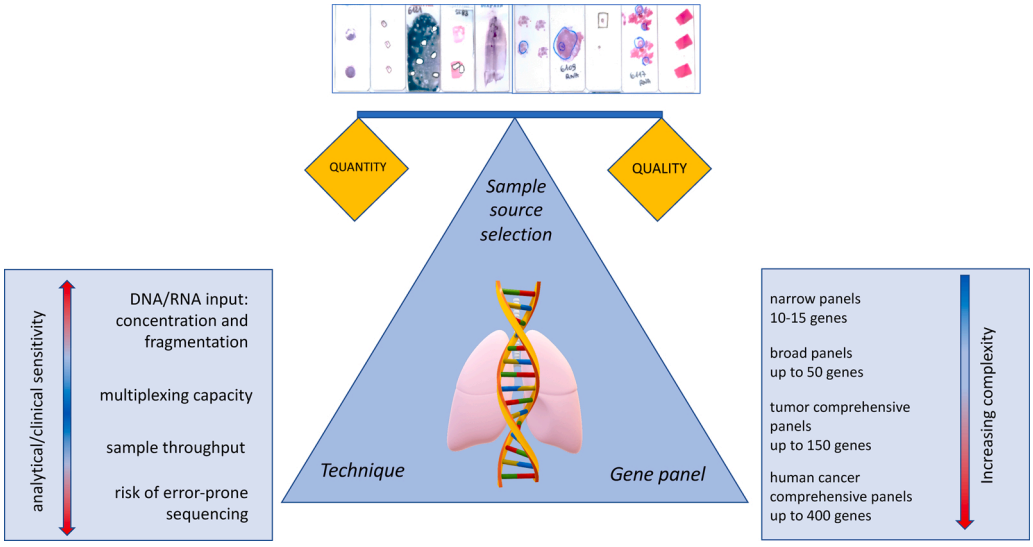

Fig. 1. NGS is fundamental for a wide molecular profiling of NSCLC and precision oncology. Its clinical power in is strictly related to the best choice of sample, technique and panel for each patient, as a magic triangle.

study, showing an optimal concordance of plasma versus tissue genotyping adopting NGS approaches (Leighl et al., 2019).

Another crucial point is represented by turnaround time. International guidelines by CAP/IASLC/AMP as well as local guidelines (Lindeman et al., 2018) recommend that molecular testing turnaround times should not exceed 10 working days. However, in real-world clinical practice, some delays in NGS turnaround time have been observed and are usually related to logistic factors (Hagemann et al., 2015).

In order to reduce time testing, in a recent retrospective study involving an Asian NSCLC cohort, authors demonstrated that upfront NGS had better time to results compared to sequential strategies (Tan et al., 2020) and similar results have been reported by Pennel et al. (Pennell et al., 2019). Interestingly, another study highlighted that sequential testing remains cost-effective only when limited to *EGFR*, *ALK* and *ROS1* analysis (Layfield et al., 2019). Ideally, comprehensive upfront DNA- and RNA- based NGS methods able to assess both mutations, rearrangements and amplifications seem the most preferable approach, in terms of time consumption and cost restraint (Drilon et al., 2015; Han et al., 2014; Tuononen et al., 2013). Concerning turnaround time, logistics is furthermore relevant as much as analytics workflows. Test ordering time, tissue collecting and selection of the most suitable sample for analysis is essential for an efficiently defined process, even in an outsourcing model of testing or if NGS testing is performed in house.

If NGS testing is performed locally, sharing equipment technology platforms among laboratories of the same institution or availability of dedicated personal equipment become crucial points to be accurately managed for optimizing reporting time and outcomes.

Delay in molecular testing may expose NSCLC patients to symptomatic progression and clinical deterioration, thus resulting in worst outcome (Blanc-Durand et al., 2021). Consequently, the adoption of "*rapid*" or "*on demand*" genotyping through single gene approach whilst running NGS parallelly, has been demonstrated to survive as a valid tool (Dagogo-Jack et al., 2018). Single gene approaches, although offering limited spectrum of mutation/rearrangements coverages, could likewise remain a rescue approach for those samples where NGS would fail due to pre-analytical tissues characteristics.

Thus, it is conceivable that molecular laboratories must be equipped with different platforms, that prove themselves to be useful also for orthogonal confirmation tests in case of challenging and rare NGS results and different assessed workflows adaptable to different clinical settings.

A decision analytic model by Pennel et al. model illustrated that moving from sequential single-gene tests or even panels of tests to broader NGS testing for patients with advanced NSCLC is the best strategy and will only become more relevant as the list of tests grows, suggesting stakeholders to consider moving to NGS as the preferred method for biomarker testing (Pennell et al., 2021).

Define the cost-effectiveness of a broad implementation of an NGS-based strategy in a specific clinical setting is still a challenging issue since case mix, throughput, expertise, logistic asset, local reimbursement, centralized laboratories policies as long as organizational impact, data confidentiality issues, availability of suitable treatments should be considered and contextualized. (Mosele et al., 2020) (Table 1).

About these topics, Pruneri et al. published a recent analysis aimed to discuss and generate evidence on one of the key drivers of decision-making in healthcare. In particular, the authors emphasized that an NGS-based approach may be less costly than a single gene testing based approach (Pruneri et al., 2021).

In terms of quality of data, the issue on pre-analytical procedures and need of standardized fixation procedures remain a key point in NGS data interpretation and diagnostic efficacy. These steps require strict internal laboratory controls (Jennings et al., 2017; Kuwata et al., 2020), along with awareness of preanalytical conditions to be managed before referring a sample to NGS testing and during NGS data analysis and reporting.

Thus, in our opinion NGS testing should be preferred over single gene testing in order to optimize tissue availability, turnaround time and costs of molecular testing.

Table 1
Overview of topics to be considered in implementing NGS in clinical practice.

Point of Strengths of NGS	Points of weakness of NGS
• Comprehensive analysis (mutations, amplifications, fusion genes) in 2 reactions (DNA-RNA- based) for targeted genes included in the panel	• More necessity of human resources and dedicated expertize required (some non-automated protocols, bioinformatics analysis of data and clinical translation of biological finds)
• Rare mutations and co-occurring alterations in emerging resistance potentially druggable, not otherwise identified in standard testing	• Pre-analytic: potential pitfall and source of failure rate (especially for RNA)
• Applicability in small clinically relevant panels or wide translational research panel.	• Incidental genomic finding to be handled (properly informed consent)
	• Reduced sensitivity for DNA-based NGS compared to RT PCR technologies
Potential point of Strengths of NGS – to be proved in the specific context of application	• Still need of some orthogonal confirmation or fast track test
• Shorter cumulative TAT compared to Single-gene strategy	• Amount of DNA/RNA required for large panels, not always available
• Cost-effectiveness	• Number of patients tested in a single run: volume needed for economic impact

However, single gene testing approaches may be adopted as orthogonal techniques useful to confirm challenging cases. A strategy for reflex molecular analysis in routine practice is proposed in algorithm in Fig. 2.

3.3. Going beyond the tissue: the role of liquid biopsy in NSCLC

As mentioned above, "*tissue is the issue*" for molecular analysis in advanced stage NSCLC patients. (Pisapia et al., 2019). In order to avoid leaving any patient behind, liquid biopsy represents a valid alternative source of tumor nucleic acids when tissue specimens are not available (Trombetta et al., 2016) and tumors are reasonably shedding. To date, from a clinical point of view, circulating tumor DNA (ctDNA) extracted from plasma is the most extensively studied and widely adopted in clinical practice (Crowley et al., 2013). However, ctDNA suffer from a limited concentration into the bloodstream (<0.005 % of the total circulating cell free DNA) and a very short half-life (about 15 min) (Pisapia et al., 2019). To this end, the IASLC established the principal role for handling this precious material (Rolfo et al., 2018).

It has been widely demonstrated the usefulness of ctDNA analysis in the analysis of clinically relevant genes in advanced stage NSCLC patients (Pisapia et al., 2017; Mezquita et al., 2020; Iaccarino et al., 2020). Regarding liquid biopsy adoption in advanced stage NSCLC patients, two different approaches have been proposed. Liquid biopsy may be adopted in association with tissue in particular in cases featuring small tissue samples with uncertain adequacy for tumor genotyping (Aggarwal et al., 2021). In alternative, it has been proposed a "blood first" approach, characterized by the adoption of liquid biopsy as the first samples for molecular analysis, in order to adopt tissue material for immunohistochemical or fluorescent in situ hybridization analysis and to confirm negative results on ctDNA samples (Leighl et al., 2019).

Currently, the term "*liquid biopsy*" has been extended to other biological fluids (saliva, urine, cerebral-spinal fluid, effusions) (Siravegna et al., 2017). In addition, even supernatants, usually discarded during cytological samples preparation demonstrated the presence of high quality tumor nucleic acids that can be useful for diagnostic molecular purposes (Roy-Chowdhuri et al., 2018; Guibert et al., 2018).

4. MTB: a new opportunity for interdisciplinarity

The unregulated use of NGS test application and new targeted drugs

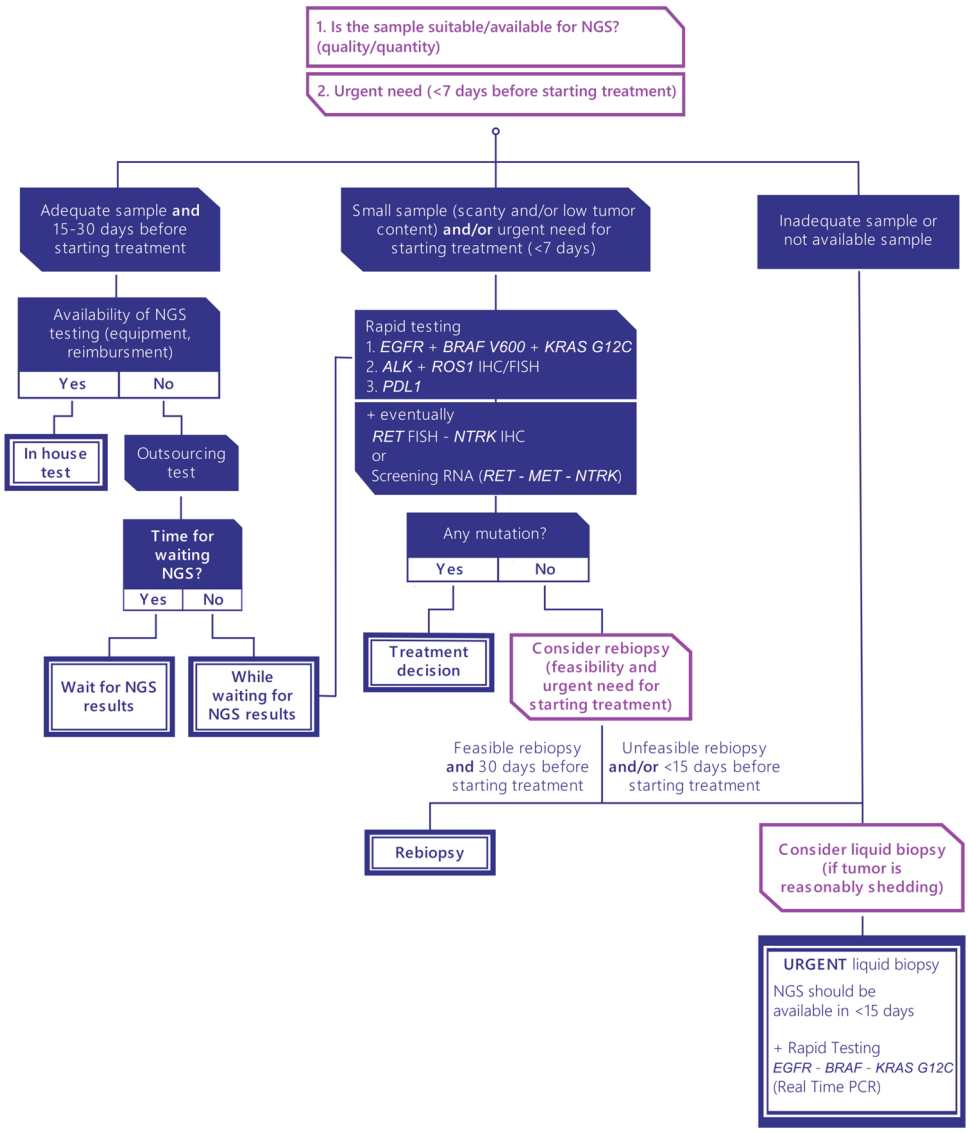

Fig. 2. Proposed algorithm for reflex molecular analysis in routine practice. Integrating sample characteristic data, knowledge about limitation and pitfall of available analytic techniques along with clinical information plays a key role in the molecular test decision.

prescription may compromise the appropriateness of novel treatments compared with standard therapies and determine a process of economical unsustainability. In order to rationalize available resources and warrant a cost-benefit ratio, an effort should be made using tests covering a minimum set of genes with sufficient sensitivity and specificity. To date, no evidence has shown a clinical benefit when wide molecular screening is offered to unselected patients, because of a moderate actionability level of molecular aberrations and because of rapid clinical worsening of the tested patients (Le Tourneau et al., 2015).

Available data underline some critical issues in the precision oncology model. Indeed, both in clinical trials (Flaherty et al., 2020) and in the real-world practice (Bonanno et al., 2020), presence of an actionable druggable target is observed in about 40 % of the overall tested population; more important, the access to the specific targeted drug was observed in about 15 % of patients. Therefore, selection criteria both for eligible patients and for molecular alterations to be tested should be identified and applied. Such alterations have been recently identified by actionability criteria according to ESCAT-ESMO (Mosele et al., 2020) and OncoKB (Chakravarty et al., 2017).

In this complex scenario, Molecular Tumor Boards (MTB) have been proposed as a governance instrument which aims to bring some "*sun into the storm*" of the new mutational model in oncology (Table 2).

MTBs should be composed by medical oncologists, hematologists, genetists, molecular biologists, pathologists, pharmacists, experts of genomic repositories and privacy rules, and upon specific request of core MTB for specific case, surgeons and radiotherapists in order to manage clinical processes, appropriateness and economical sustainability.

MTBs discussion should include a whole patient clinical history review in presence of different medical specialties to allow the establishment of a patient-customized diagnostic and treatment plan with the ultimate goal of allocating the right diagnostic and treatment available resources for each patient (Kato et al., 2020).

Different drugs access are currently available, such as named patient use, expanded access program or off-label indications, and these may be eligible or not to MTB discussion according to institutional procedures of reference compared to second-level centers.

The interdisciplinary nature of MTB also allows for a comprehensive education on clinically relevant emerging molecular biomarkers, associated treatments and potential strategies for early access, to the whole board. Moreover, topics like samples type, availability and management, choice of the test for the specific clinical indication are critical for predictive biomarkers testing and can be addressed thus leading to a

Table 2
Data of prevalence and actionability of main NSCLC alterations, according with ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) (Mosele et al., 2020) and European regulatory agencies (EMA). Green cells: ESMO ESCAT IA (bright)-IB (light) targets with reimbursed drug availability in clinical practice; yellow cells: ESMO ESCAT IB-C (bright) and IIB (light) targets with an available named patient use or expanded access program; red cells: ESMO ESCAT IIB (light) or not available ESMO ESCAT (dark) targets to be discussed at MTB. (For interpretation of the references to colour in this Table legend, the reader is referred to the web version of this article).

	Prevalence	Available drug	Request for expanded access program or named patient use (refer to MTB, according to insitution procedures)	Actionability to be defined search for off Label or clinical trials
EGFR exon19 - deletions *EGFR* exon21 - L858R	15%	ESMO ESCAT IA gefitinib erlotinib afatinib osimertinib		
EGFR exon 18 - G719x EGFR exon 20 – S768I EGFR exon21 - L861Q	2-3%	ESMO ESCAT IB afatinib osimertinib		
EGFR exon 20 - T790M	50% EGFR mutant pretreated- NSCLC	ESMO ESCAT IB (II line of treatment) osimertinib		
EGFR exon 20 - insertions	2%		ESMO ESCAT IIB poziotinib mobocertinib amivantanab	
EGFR exon 20 - C797S	10-30% EGFR mutant pretreated- NSCLC			refer to MTB (resistance mechanism)
BRAF V600E	2%	ESMO ESCAT IB dabrafenib+trametinib		
ALK - fusion	5%	ESMO ESCAT IA crizotinib ceritinib alectinib brigatinib lorlatinib		
ALK - mutations				refer to MTB (resistance mechanism)
ROS1 - fusion	1-2%	ESMO ESCAT IB crizotinib lorlatinib		
ROS1 - mutations				refer to MTB (resistance mechanism)
MET exon 14 skipping	3%		ESMO ESCAT IB capmatinib tepotinib	
MET amplification	3%			ESMO ESCAT IIB refer to MTB (resistance mechanism)
RET fusion	1-2%		ESMO ESCAT IC selpercatinib pralsetinib	refer to MTB
ERBB2 duplications/ insertions	2-5%			refer to MTB
ERBB2 amplification				ESMO ESCAT IIB refer to MTB
NTRK fusions	0.2-3%		ESMO ESCAT IC entrectinib larotrectinib	
KRAS G12C	12%		ESMO ESCAT IB sotorasib	

testing protocol that takes into considerations all aspects of samples management and testing methodologies to provide the clinicians an actionable result.

Probably artificial intelligence tools will become fundamental for big data management in genomics, along with interrogation of several databases for multiple clinical data correlation and therapy options, worldwide available.

Considering NGS testing reporting, despite emerging issue of standardization (Lubin et al., 2017; Li et al., 2017), significant differences exist across laboratories with no consistency on which alterations need to be reported to the requesting physicians. Even if a common practice, this needs to be discussed in a multidisciplinary forum where all aspects of treatment management are taken into considerations for the maximum benefit for the patients and the right investment of the National Health System resources. It is paramount then to offer extensive NGS profiling to evaluate all possible molecular alterations in light of nationally approved drugs, of the possibility of early access strategies and of the presence of clinical trials in the country and/or elsewhere where the patients can be easily enrolled.

This allows offering molecular profiling only to those suitable candidates meeting specific criteria (life expectation, tumor rarity, responsiveness to prior treatments and sample availability) (Luchini et al., 2020).

5. Conclusion

In the era Precision Medicine era, NSCLC represents one of the more challenging and rich playground both for clinical therapeutic chances and for biological and technical aspects. Opportunities, pitfalls and gray areas of innovative diagnostic approaches could be overcome and handled in the near future within the context of new organizational models such as MTBs. As long as these new arrangements catch on and spread, continuous discussion between pneumologist, pathologist, molecular biologist and oncologist should be promoted and implemented in NSCLC patients management.

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