Portuguese Consensus Recommendations for Next-Generation Sequencing of Lung Cancer, Rare Tumors, and Cancers of Unknown Primary Origin in Clinical Practice

Recomendações Portuguesas para a Utilização de Sequenciação de Nova Geração na Prática Clínica em Tumores do Pulmão, Raros e de Origem Primária Desconhecida

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ABSTRACT

Next-generation sequencing (NGS) has been implemented in clinical oncology for diagnosis, prognosis, and therapeutic guidance. Among the various NGS applications in molecular oncology, we focused on the following topics: laboratory standards for targeted gene panels (somatic mutations) and therapeutic guidance based on NGS of lung cancer and rare cancers, namely sarcomas and cancers of unknown primary. Multiple quality control checkpoints should be addressed in the pre-analytical phase for good quality and interpretation of the NGS results. It includes tumor size and cellularity, tissue processing and decalcification, tumor fraction, tumor viability, fixatives, and staining. Communication between clinicians and laboratory support is also essential. In lung cancer, all patients with non-squamous non-small cell lung cancer should be tested with a NGS panel, and it should include not only genes with approved targeted therapies (*ALK*, *BRAF*, *EGFR*, *MET*, *NTRK*, *RET*, and *ROS1*) but also genes with potentially actionable genomic alterations (*HER2* and *KRAS*). Since there is a lack of extensive knowledge regarding the use of NGS in rare tumors, performing comprehensive genomic profiling panels to better manage the disease is recommended. Moreover, other patients with other incurable solid tumors may benefit from being included in biomarker-driven clinical trials. Multidisciplinary tumor boards with the participation of experts with the ability to integrate genomic profiling data are essential to tailor the best strategy for each patient. Considering that there are no national guidelines, this article aims to guide laboratory and clinical practice for the use of NGS in the context of lung cancer, rare tumors, and cancer of unknown primary in Portugal.

Keywords: High-Throughput Nucleotide Sequencing; Lung Neoplasms/genetics; Neoplasms, Unknown Primary/genetics; Sarcoma/genetics

RESUMO

Na área da oncologia clínica, a sequenciação de nova geração (NGS) foi implementada com o objetivo de contribuir para o diagnóstico, prognóstico e orientação terapêutica. A utilização de NGS em oncologia molecular é vasta, focalizando-se estas recomendações nas: normas laboratoriais para painéis genéticos direcionados (mutações somáticas) e na orientação terapêutica baseada em NGS de cancro do pulmão e cancros raros, nomeadamente sarcomas e cancros de origem desconhecida. Para que sejam obtidos resultados de NGS com a qualidade que permita a sua correta interpretação, devem ser abordados múltiplos controlos de qualidade na fase pré-analítica que disponibilizem informação sobre o tamanho e celularidade do tumor, processamento e descalcificação de tecidos, fração tumoral, viabilidade do tumor, fixadores e coloração utilizados. A comunicação entre os diferentes intervenientes no processo, em particular entre os clínicos e o laboratório também contribui, de forma inequívoca, para a interpretação dos resultados de NGS. Todos os doentes com cancro do pulmão de não pequenas células não escamoso devem ser testados com um painel de NGS, que deve incluir não só genes com terapias dirigidas aprovadas (*ALK*, *BRAF*, *EGFR*, *MET*, *NTRK*, *RET* e *ROS1*), mas também genes com alterações genómicas identificadas como potenciais alvos terapêuticos (*HER2* e *KRAS*). Dada a escassez de evidência científica sobre a utilização de NGS em tumores raros, recomenda-se a realização de painéis genómicos abrangentes que poderão

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contribuir para uma melhor gestão da doença. Adicionalmente, outros doentes, com outros tumores sólidos incuráveis, podem beneficiar da inclusão em ensaios clínicos orientados por biomarcadores. A realização de reuniões multidisciplinares com a participação de diferentes especialistas capazes de integrar dados dos perfis genómicos são fundamentais para a escolha da melhor estratégia para cada doente. Considerando que não existem recomendações nacionais, este artigo visa orientar a prática laboratorial e clínica para a utilização de NGS em tumores do pulmão, raros e cancros de origem primária desconhecida em Portugal.
Palavras-chave: Neoplasias Primárias Desconhecidas/genética; Neoplasias do Pulmão/genética; Sarcoma/genética; Sequenciação de Nucleotídeos em Larga Escala

INTRODUCTION

Molecular mechanisms that can impact tumor initiation, growth, progression, and metastasis[1] have become clinically valuable with the advance of targeted therapies and diagnostic tools, contributing to precision medicine.[2]

When assessed by hematoxylin-eosin staining, the pathologist assesses the morphology of the tumor tissue and the pattern of expression in order to provide an overview of tissue characteristics.[3] Although some biomarkers can be assessed by immunohistochemistry (IHC) with a predictive result (e.g., ER, PR, HER2), the detailed molecular characterization is not entirely clarified this way, and developments in sequencing techniques allow for a more detailed understanding of the tumor molecular mechanisms.[3]

Next-generation sequencing (NGS) is a technology that decodes genetic information easier, faster, and at a lower cost compared to than Sanger sequencing. The term NGS includes a group of technologies, also called *massively parallel sequencing*, that share the ability to simultaneously analyze multiple genomic regions through data capture from millions of sequencing reactions.[4-7] This technique is linked with bioinformatic tools which are essential for analyzing the vast amount of generated data.[8] These data can be used to support patient management.[3]

NGS is a widely accepted molecular biology technique that can analyze DNA and RNA, contributing to an accurate diagnosis and the detection of actionable mutations that sensitize the tumor to specific therapies.[2,9] Its applicability ranges from clinical research, usually with broader approaches like whole-genome, whole-exome, and transcriptome, to more focused clinical applications by targeted gene panels evaluation.[2] The NGS workflow comprises three main processes: library preparation, sequencing, and bioinformatics data analysis.[10]

Among the various NGS applications in molecular oncology, we will focus on laboratory standards for targeted gene panels (somatic mutations) and their use in the diagnosis, therapeutic guidance, and prognosis of lung carcinomas and rare tumors such as sarcomas and cancers of unknown primary.

The aim of this article is to provide recommendations for the use of NGS in Portuguese clinical practice since there are currently no national guidelines.

Practical recommendations for NGS from an expert group

NGS is a relatively new field in solid tumors, and therefore few guidelines are currently available,[11,12] including the latest 2020 ESMO guidelines.[13] The genes to be tested depend on the testing purpose and will also rely on the availability of targeted treatments and reimbursement schemes that are different in each country.[13] The elected method should be an assay detecting clinically actionable genomic alterations, defined by the clinical diagnosis and/or availability of targeted drug therapies. Genomic alterations

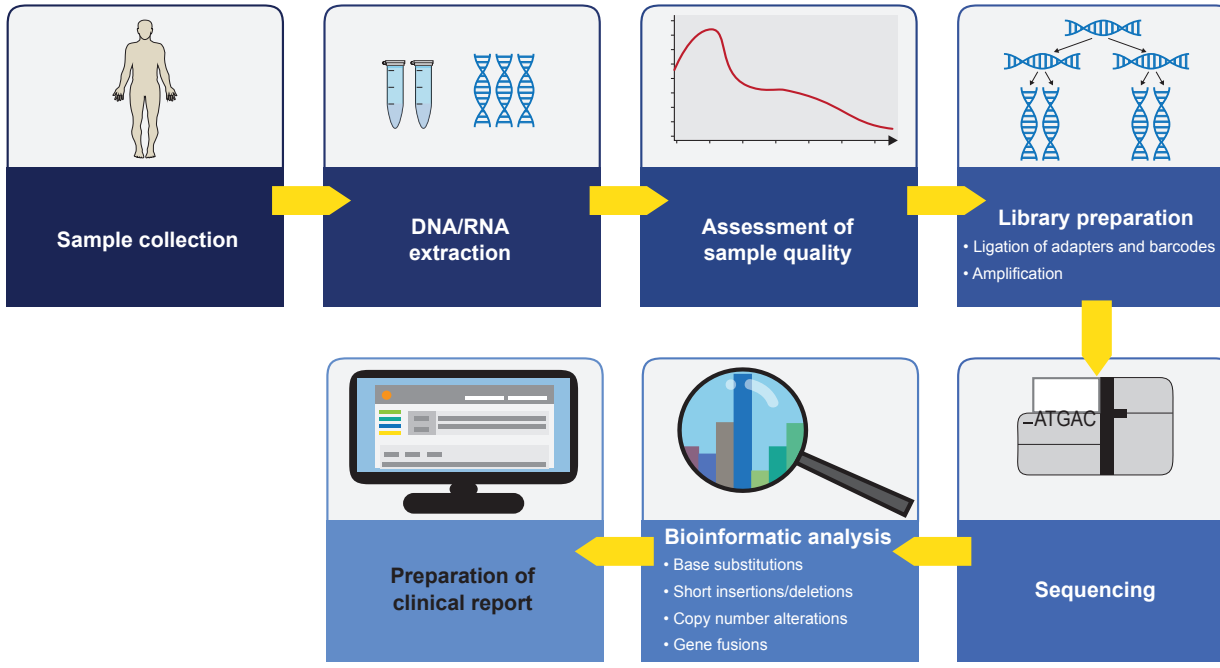

Figure 1 – Next-generation sequencing workflow

associated with acquired resistance to target-based agents are also being included in genomic panels.[11]

Although NGS is a powerful technique to assess clinically relevant genetic alterations in tumors, some issues need to be addressed in the NGS workflow (Fig. 1), namely in its implementation, laboratory standards, and data interpretation.

NGS

What pre-analytical conditions should be met to perform NGS?

Recommendation: The multiple quality control checkpoints in the pre-analytical phase should include tumor size and cellularity, tissue processing and decalcification, tumor fraction, tumor viability, and fixatives and staining. In a patient with multiple specimens available from different timepoints, the most recent one should be used for NGS. Morphologic control is one of the leading quality control checkpoints that could significantly impact the reliability and interpretation of NGS results. Testing procedures needed to be validated locally and that include defining minimum DNA input and minimum tumor cell content. Multidisciplinary communication is essential for the optimization of specimen acquisition and processing.

The success of molecular diagnostics in oncology depends on various factors. One of the most important is the proper selection of tumor samples, the quantity, the quality of the tumor specimen, and tumor cellularity.[14,15] Every stage from collecting the specimen to its analysis can influence NGS results, treatment decisions, and clinical outcomes. One of the main challenges in the molecular oncology of solid tumors is the quality of nucleic acids, as the process of formalin fixation of specimens could compromise DNA and RNA integrity through chemical cross-linking of protein and nucleic acids.[16,17] Nevertheless, with the improvement of methods of processing formalin-fixed paraffin-embedded (FFPE) material, that is no longer a limitation for the use of NGS as routine testing in these samples.[11,18] Moreover, nucleic acid yield could be low due to limited tissue obtained through fine-needle aspiration and core-needle biopsy.[15]

Therefore, there is a need to standardize pre-analytical conditions to ensure the accuracy and reliability of the results, which increases the credibility and the use of NGS in clinical practice.

The pre-analytical factors associated with the success of NGS are the cold ischemia time, tissue fixation, processing and storage, sample size and cellularity, tumor cell fraction, tumor viability, use of decalcification, and other factors such as the presence of blood and mucin.[15]

Type of procedure: Tumor samples can be obtained by surgical resection, endobronchial biopsy excisions, fine-needle aspiration, and core-needle biopsy.[15] The first is associated with a larger tumor section and higher DNA yield.[15,19]

Tumor site: After adjusting for tumor size and, in the absence of decalcification, the various solid tumors, sampled by the different procedures showed similar NGS success rates.[19]

Tissue processing and storage: Direct preservation of tissue specimens ideally follows a controlled and defined process, such as formalin (buffered formalin at 10%) fixation beginning immediately after removal.[20,21] The volume of buffered formalin should be adequate since the fixation time is dependent on the specimen volume (minimum six hours/maximum 72 hours).[21,22] The preferred tissue conservation methods to preserve the molecular profiles of cells and cytology samples are FFPE tumor tissue and cryopreservation (-80ºC to -190ºC).[23,24]

Tumor size and cellularity: One of the most important pre-analytical requirements for a reliable NGS assay is the specimen's quantity and quality. The obtained material should be sufficient for a correct and accurate morphologic diagnosis and control and posterior biomarker analysis through NGS.[14,24] The size of the tumor area and the number of viable tumor cells will determine the DNA yield.[15,19] The morphological diagnosis and cellularity estimation in tissue and cytological material are vital to the correct execution of NGS.[11] The sample should include as many tumor cells as possible, but NGS works with very low tumor cell content. Each laboratory should define its threshold. If a sample is below the defined threshold and there is a negative result, then there is a risk of being a false negative and that should be clearly stated in the report.

A molecular pathologist, using macrodissection, should guide the nucleic-acid extraction area, marking tumor tissue and normal tissue, thus increasing the yield of the sequencing technique. It requires specific staff training and institutions should have dedicated pathologists to perform this task.[14,15]

Tumor fraction: Another important point to consider is the proportion of tumor cells in the specimen, the so-called tumor fraction or tumor purity.[14] Diverse NGS assays could have different tumor fraction requirements due to different NGS platforms' distinctive technical sensitivities. Ideally, a NGS assay should be able to detect a mutation with a variant allele frequency (VAF) as low as 5%. Given the heterozygous nature of somatic mutations in most tumors and the possibility of genetic intratumor heterogeneity, the selected specimen area should have a tumor cell fraction of at least 20%.[15,25-29] In small specimens, such as core needle biopsies and cytology samples, NGS could be less successful than in larger samples such as the ones obtained from resection and excisions. Other types of samples may be acceptable as long as they are validated locally in the laboratory.

Tumor viability: The viability of the tumor tissue is essential for the success of NGS. Necrosis can occur among the different tumor types and should be carefully analyzed and interpreted.[15]

Decalcification of bone specimens: Before decalcification, it is necessary to perform an adequate tissue fixation.[15] Only bony samples that undergo formic-acid- or EDTA-based decalcification procedures are adequate for both

morphologic analysis and NGS.[30] A solution consisting of formic acid (88% formic acid diluted 1:10 in distilled water) with constant stirring can be used for tissue decalcification after formalin fixation. For checking the decalcification process, x-ray analysis may be performed daily until decalcification is demonstrated by radiographic evidence. For neutralization of the decalcified block, a solution of 0.3% ammonium hydroxide in 80% ethyl alcohol can be used.[19]

How to implement NGS in a diagnostic laboratory?

Recommendation: Quality control should be implemented for all pre-analytic, analytic, and interpretation procedures. If that is not possible, an external molecular biology laboratory is the best option.

It is essential to test and validate the method before the implementation of any NGS-based diagnostic test. Besides all the pre-analytical conditions included in the previous section that should be under periodical quality control assessment, the assay's adequacy to cover clinically relevant variants to a sufficient depth for variant calling, as well as optimization of the bioinformatics pipeline to detect relevant mutations, are essential.[11,31] This typically includes an assessment of sensitivity, specificity, and reproducibility, in addition to other performance characteristics as required by the relevant laboratory-certifying authority. The performance characteristics of NGS assays can be readily determined for the most common somatic alterations. However, the reliability of detection for uncommon somatic alterations or specific categories of mutations, such as large insertion-deletions (indels) or certain chromosomal alterations may be more challenging to establish. Hence, laboratories should have procedures to verify any unexpected results, namely those that are discordant with other results, equivocal, or of compromised confidence. These include obtaining alternative samples, testing with an orthogonal methodology with a different selectivity of the primary NGS method, or testing in another laboratory. If the local molecular biology laboratory does not have sufficient capacity, an external NGS laboratory with quality control is the best option.

Which information should be given to the molecular biology laboratory?

Recommendation: Information to be given to the molecular biology laboratory should include:

1. Patient identification, with at least two identifiers.
2. Diagnosis or potential diagnosis, with staging information, if available.
3. Test results from other previously performed molecular tests, if available.
4. Specimen information, type of specimen, tumor cell content.
5. The objective of the test:
   a. Differential diagnosis?
   b. Need to distinguish between two primary tumors or between one primary tumor and one metastasis?
   c. Therapy decision at diagnosis?
   d. Therapy decision after resistance acquisition to previous targeted therapy?
   (describing previous therapies and their sequence)
   e. More comprehensive biomarker testing for possible inclusion in a clinical trial or off-label therapy?

For optimizing the NGS analysis and consequently obtaining better results for patients, communication between the clinic, pathology laboratory and molecular biology laboratory is key.

Which information should be included in the NGS report sent to the clinician?

Recommendation: The report should be standardized and include all the essential information for the correct interpretation of the results. An example of an appropriate report for clinicians is included in the Appendix 1 (Appendix 1: https://www.actamedicaportuguesa.com/revista/index.php/amp/article/view/17680/Appendix_01.pdf).

There are international diagnostic standards such as ISO 15189 and guidelines that should be followed to report the results.[11,12,31-34] The length of the report should not exceed one page, be easily read, and contain the following essential information: patient identification, sample type, tissue/tumor type, tissue sample identification, the restatement of the clinical question, percentage of tumor sample content used for NGS, depth coverage, NGS method used, sensitivity of the method, reference sequences for tested genes, results using the Human Genome Variation Society mutation nomenclature, how/where additional information can be obtained, biological and clinical interpretation of the results and conclusion.[11] Information on the variant allele frequencies (VAFs) may also be provided. Results and conclusions according to the clinical question should be highlighted. Moreover, the results section should be divided into clinical, clinical trials, and research domains.[11] Clinical domain: variants with a current approved therapeutic indication or used for diagnosis, prognosis, or therapeutic monitoring; clinical trials domain: variants that can predict response to new drugs and allow the enrollment in a clinical trial; research domain: variants of uncertain clinical significance, with an unknown biological role in oncogenesis.[11]

Decisions based on the NGS results should consider all other pathology and clinical data and eventually be discussed in a multidisciplinary or molecular tumor board (MTB) context.

Lung cancer

Lung cancers are classified into two main histological types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC has a higher prevalence accounting for 85% of bronchogenic carcinomas.[29,35] Among NSCLC, adenocarcinoma and squamous cell carcinoma

are the most common histologic types.[35]

In which histological type of lung cancers should NGS be performed?

Recommendation: Due to the expanding number of actionable genomic alterations, all non-squamous NSCLC should be tested with a NGS panel. Exceptions may be considered in the multidisciplinary meeting.

NGS allows the identification of genomic alterations down to single-base-pair resolution with a high level of precision and accuracy.[35] This leads to therapeutic progress and the development of new drugs.[36] Approximately 69% of patients with NSCLC could have a potentially actionable molecular target.[37]

In adenocarcinoma, the most commonly mutated oncogenes are *KRAS*, *EGFR, PIK3CA*, *MET,* and *BRAF*; the mutated tumor suppressors are *TP53*, *STK11*, *KEAP*, *NF1*, *RB1,* and *CDKN2A*.[38] Gene fusion and rearrangement of *ALK*, *ROS1*, *NTRK1*, *NRG1*, *FGFR4*, *ERBB4,* and *RET* are also important modifications in lung adenocarcinoma, with *ALK*, *ROS1*, *NTRK* and *RET* already having approved targeted therapies.[38-43] Squamous-cell carcinoma is characterized by fewer mutations in genes coding for receptor tyrosine kinase and a higher frequency of mutations in tumor suppressor genes such as *TP53*, *PTEN*, *NOTCH1,* and *RB1*.[44] FGFR-gene family rearrangements have been reported in squamous-cell cancer and can be targetable.[45] While the known adenocarcinoma targetable alterations are well characterized, personalized medicine in the treatment of squamous cell carcinoma is lagging far behind adenocarcinoma and NGS could be a powerful technique to identify genetic alterations and allow easy integration in clinical practice.[46]

At what disease stage should NGS be performed?

Recommendation: All patients with advanced disease must be tested. Patients with limited disease, candidates for adjuvant targeted therapies e.g., *EGFR*, should be considered for testing. Moreover, patients with limited disease are at high risk of progression; thus, we recommend that earlier testing, at diagnosis, may be considered. At the time of progression, in the setting of targeted therapy, a new NGS test is recommended.

The recommended treatment for patients with stage I-II NSCLC is surgery complemented by adjuvant chemotherapy for some patients.[47] For patients with locally advanced (stage IIIA-B) unresectable tumors, stereotactic body radiotherapy with concurrent chemotherapy delivery is recommended.[47] More recently, durvalumab (anti-PD-L1 antibody) has been approved as maintenance therapy in this setting.[48,49] In these earlier stages of the disease, there is currently no role for targeted therapy outside clinical trials. However, this is expected to change shortly, with the ADAURA trial results showing a disease-free survival (DFS) advantage for using osimertinib in stages IB-IIIA EGFR mutation-positive NSCLC.[50] Despite these treatment advances, patients diagnosed at this stage are at high risk of relapse and survival remains low.[51]

At the time of progression, genomic-guided treatments are becoming increasingly relevant. Although tissue re-biopsy to repeat molecular testing is not always feasible, blood liquid biopsy is likely to become a validated routine alternative.

Which are the genes that should be included in the NGS panel?

Recommendation: The initial testing NGS panel should include genes with clinical relevance:

- genes with approved targeted therapies: *ALK, BRAF, EGFR, MET, NTRK, RET and ROS1*
- other oncodrivers: *HER2 and KRAS.*

Recently published ESMO guidelines for NGS recommend performing tumor multigene NGS to assess level I genomic alterations.[13] Level I genomic alterations include *EGFR*, *M*ET, and *BRAF* mutations and *ALK*, *ROS1*, *NTRK,* and *RET* fusions.[13] Additionally, larger panels can be used considering the total cost burden strategy, assuming an accurate ranking of alterations is reported. For clinical research centers, performing multigene sequencing panels in molecular screening programs is highly recommended. It will increase access to innovative drugs and speed up clinical research.[13]

EGFR somatic mutations have been reported in 20% of Caucasians with NSCLC, and therapies with tyrosine kinase inhibitor (TKI) targeting EGFR were pioneers in the era of targeted therapy.[52,53] In Portuguese patients with metastatic NSCLC, 14% harbor *EGFR* somatic mutations.[54] Among the first-generation EGFR-TKI are gefitinib and erlotinib which have been used as first-line therapy in patients harboring *EGFR* mutations (exon 21 L858R and exon 19 deletions).[55,56] Second generation EGFR-TKI such as afatinib and dacomitinib inhibit the four members of the ERBB family.[57,58] Resistance to first- and second-generation EGFR-TKI is common amongst patients and is mediated by the T790M resistance mutation in half of them.[53] Third-generation irreversible EGFR-TKI targeted therapy, such as osimertinib, is selective for EGFR-TKI sensitizing and T790M resistance mutations.[59-61] Treatment with osimertinib as second-line therapy requires the detection of the *EGFR T790M* mutation in a liquid biopsy or in a tissue re-biopsy. If a liquid biopsy is chosen as the initial test, and if negative, a tissue re-biopsy should be performed due to sensitivity limitations, if feasible.[62] The search for mechanisms of resistance to EGFR-TKI therapy should not be limited to *T790M EGFR* mutations, as other genes are involved as well, including *HER2, BRAF* (V600E), *KRAS* (G12D/C, A146T), and *PIK3CA* mutations, SPTBN1-ALK fusions and MET amplifications.[53] Moreover, as resistance to osimertinib also occurs, other mutations in the *EGFR* gene, such as C797S, should also be searched for. Therefore, NGS testing in this context should be performed.[42]

ALK rearrangements occur in 5% of lung adenocarcinomas, namely in non-smoker younger adults.[53] Crizotinib was the first ALK inhibitor with identified resistance emerging from two major secondary mutations, L1196M and C1156Y.[53,63] Alectinib is a second-generation ALK inhibitor with high selectivity for *ALK* rearrangements that overcomes these two mutations.[64] Brigatinib, ceritinib, and lorlatinib are also specific inhibitors that can overcome resistance through other secondary mutations.[42] Thus, in case of resistance, and although it is not included in the international guidelines[62], tissue re-biopsy or liquid biopsy for NGS analysis can be a potential tool for the choice of a subsequent ALK inhibitor.[42,53,65-67]

ROS1 rearrangements are less common than *ALK* rearrangements and share approximately 70% of homology.[53] Crizotinib has also shown activity in *ROS1* rearrangements and is approved for this indication.[42,68] Entrectinib is a ROS1 inhibitor with the ability to penetrate and remain in the central nervous system. It is approved for the treatment of advanced NSCLC in *ROS1* positive patients. Data from entrectinib and crizotinib approvals plus the ongoing trials with other inhibitors, highlight the need to test for *ROS1* fusion in NSCLC to broaden the therapeutic options in these patients.[69]

NGS is a validated technique for sequencing *ALK* and *ROS1* rearrangements, with an advantage over immunohistochemistry and fluorescence *in situ* hybridization (FISH) for detecting potential actionable molecular alterations.[70]

Among the 1% - 2% of advanced NSCLCs with *BRAF V600E* mutation, 85% are adenocarcinomas.[71] The combination of dabrafenib with trametinib, a *MEK* inhibitor, demonstrated good results in previously untreated patients.[72]

In *NTRK* fusion-positive NSCLC tumors, larotrectinib and entrectinib have shown effective results and are approved for the treatment of these patients.[73] *NTRK* gene fusions are present in 0.2% - 3.3% of NSCLC tumors and the approval was based on basket trials that included different solid malignancies.[74-76]

RET rearrangements are commonly found in younger patients below 60 years old, non-smokers, or former light smokers.[77] So far, multikinase inhibitors (like cabozantinib, lenvatinib, and vandetanib) have been used *off label* for *RET*-positive NSCLC. Nevertheless, both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have already approved more selectively targeted and potentially effective TKIs, like pralsetinib and selpercatinib.[78,79]

Mutations and amplification of *MET* are commonly found in elderly patients and non-smokers, and therapies such as crizotinib, capmatinib, and tepotinib have shown effectiveness in treating these patients.[80-82] Thus, *MET* should be included in earlier NGS panel tests.[83]

Besides these seven genes implemented in clinical practice, other target driver oncogenes have been studied in NSCLC, such as *KRAS* mutations and *HER2* mutations.[42,53,83-85]

KRAS is the most commonly mutated oncogene in NSCLC (in approximately 30% of the patients), and although it is not considered in the ESMO recommendations, it should be included in all NGS genomic panels since it is a prognostic biomarker.[62,86] Moreover, *KRAS* mutations when associated with *TP53* and *STK11* co-mutations may be vulnerable to immunotherapy approaches.[84,87,88] Aside from that, sotorasib and adagrasib, two novel *KRAS* G12C small molecule inhibitors, showed overall early promising results with antitumor activity and a manageable safety profile in heavily pre-treated patients with NSCLC.[89,90]

HER2 mutation in NSCLC is an oncologic driver mutation that is a promising target for treating patients with advanced disease that progressed on or after platinum-based therapy. Trastuzumab deruxtecan has promising results in this context.[85]

Repotrectinib is a novel next-generation ROS1-TKI inhibitor with promising results, namely high activity in the central nervous system, in ROS1 positive and recalcitrant crizotinib-resistant G2032R mutation NSCLC.[91]

A summary of the approved therapies and under clinical investigation is summarized in Table 1.

Should an NGS-based liquid biopsy be performed in lung cancer?

Recommendation: Liquid biopsy for NGS evaluation of actionable mutations can be performed, if validated, at diagnosis or in cases of resistance/progression under targeted therapy when a tissue biopsy cannot be performed. Moreover, liquid biopsies can complement tissue biopsies, providing an in-depth idea of tumor heterogeneity.

Tissue biopsies remain irreplaceable as the basis for histopathological diagnosis. Liquid biopsies (circulating cell-free tumor DNA) are routinely used to detect resistance mutations upon progression on TKIs, such as *EGFR* T790M mutation after first-line therapy with EGFR inhibitor, to address intra-tumor heterogeneity and also in the detection of new mutations.[110] In the context of diagnosis, liquid biopsy is recommended in the following specific situations[36,111]:

- tissue biopsy is not safe, contraindicated, or declined by the patient;
- the quantity and quality of tumor tissue is not enough for a correct molecular diagnosis;
- delay is expected to occur in the availability of tumor tissue.

In case of resistance to first or second-line EGFR-TKI, NGS using circulating cell-free DNA has shown high sensitivity, identifying multiple resistance alterations.[112,113]

Recently, the FDA approved a pan-cancer cell-free DNA (cfDNA) based comprehensive genomic profiling assay for cancers of solid origin.[114] cfDNA is isolated from plasma derived from anti-coagulated peripheral blood of cancer patients collected in specific tubes.[114] Apart from the *in vitro* diagnosis of a high number of target genes and although these biomarkers are currently not validated in lung cancer, these tests also allow the assessment of TMB (tumor mutational burden), MSI (microsatellite instability), and tumor

Table 1 – Targeted therapies for genomic alterations in advanced NSCLC

Genomic alteration	Targeted therapy
Approved	
***EGFR*-activating mutations**	Gefitinib[92]
	Erlotinib[93]
	Afatinib[94]
	Dacomitinib[95]
	Osimertinib[96]
***ALK* translocation and rearrangements**	Crizotinib[97]
	Alectinib[98]
	Ceritinib[99]
	Brigatinib[100]
	Lorlatinib[101]
***ROS1* translocation and rearrangements**	Crizotinib[97]
	Entrectinib[102]
***BRAF V600E* mutation**	Dabrafenib with trametinib[103,104]
***NTRK* fusions**	Larotrectinib[105]
	Entrectinib[102]
***MET* mutation and amplification**	Capmatinib[106]
	Tepotinib[82]
***RET* translocation and rearrangements**	Pralsetinib[107]
	Selpercatinib[108]
In clinical trials	
***HER2* mutation**	Trastuzumab deruxtecan[85]
***KRAS* mutation**	Sotorasib[89]
	Adagrasib[109]
***ROS1* translocation and mutation**	Repotrectinib[91]

fraction values.

Also, in case of resistance to an ALK inhibitor, tissue re-biopsy or liquid biopsy for NGS analysis enables the evaluation of the resistance mutation profile to first-line therapy. However, this is currently not really necessary for the choice of the second-line ALK inhibitor.[42,53,65-67]

Although some reports show some discrepancies between tissue- and liquid- biopsies, this could be due to the intrinsic differences of the sample, assays, bioinformatics tools, and tumor heterogeneity.[8] Each liquid biopsy test must be validated. There are already commercially approved NGS-based liquid biopsy tests based on a clinically validated comprehensive cell-free DNA analysis that identifies recommended biomarkers at the rate as high as standard-of-care tissue genotyping, with high tissue concordance.[115,116]

Rare tumors

Rare Cancers Europe defines rare cancers as occurring in fewer than six out of 100 000 people each year.[117] This type of tumor includes, among others, sarcomas and cancers of unknown primary and are more difficult to prevent, diagnose and treat than other types of cancer.[118] Tissue samples from 5945 patients with refractory and underexplored cancer types were analyzed in the clinical trial National Cancer Institute Molecular Analysis for Therapy Choice (NCI-MATCH). The results showed that actionable genomic alterations were present in 11.9% of samples and resistance mutations were present in 71.3% of the specimens. The authors conclude that NGS is feasible and can help sort patients to investigational therapy in genetically complex tumors.[119]

Sarcomas

Sarcomas are rare mesenchymal malignancies that include at least 100 different subtypes.[120,121] Diagnosis is based on morphological, immunohistochemical, and molecular characterization, although a differential diagnosis is often difficult.[121] In the most recent years, a significant number of translocations have been described, helping in the diagnosis and characterization of sarcomas. Nevertheless, in clinical practice, targeted therapies are still poorly implemented, except for some subtypes such as gastrointestinal stromal tumor (GIST) and the use of pazopanib in soft tissue sarcomas.[122-124]

How can NGS be used in sarcomas?

Recommendation: NGS is a valuable tool in the management of sarcomas. Comprehensive genomic profiling NGS panels are already available, but they are expensive. More data is essential and therefore patients should be included in biomarker-driven clinical trials when available. These patients should be managed in centers with a considerable number of patients/year and multidisciplinary teams.

A NGS panel containing probes for 87 fusion genes and seven genes with frequent copy number alteration was designed and applied on 113 DNA samples extracted from FFPE samples of soft-tissue and bone sarcomas. FISH or RT-PCR had already analyzed these samples and the results showed that NGS is a feasible and cost-effective approach allowing to test a wide range of genomic aberrations at the same time, which can be very useful for the differential diagnosis of sarcomas.[121]

Current therapy includes cytotoxic chemotherapy drugs and tyrosine kinase inhibitors such as pazopanib for most patients with metastatic sarcomas.[125] Other approved targeted therapies for actionable mutations are imatinib, sunitinib, and regorafenib for GIST and imatinib for dermatofibrosarcoma protuberans.[122,126]

A recent study involving 133 tumor samples from patients diagnosed with different types of sarcomas analyzed over 400 cancer-related genes and found that most mutations are in genes related with the cell cycle, including *TP53*, *CDKN2A/B* and *RB1,* with 75 mutations occurring in targetable genes. Tumor mutational burden and microsatellite instability were generally low.[125] Another study using comprehensive genomic profiling also showed that among different types of sarcoma such as leiomyosarcoma, dedifferentiated liposarcoma, osteosarcoma, well-differentiated liposarcoma, carcinosarcoma and rhabdomyosarcoma, 93% of patients had at least one genomic alteration with a mean of six mutations per patient.[122]

Besides actionable mutations, chromosomal translocations and fusion genes were common among different types of sarcomas, such as rhabdomyosarcoma, Ewing's sarcoma, synovial sarcoma, and liposarcoma.[127] The resulting chimeras have altered functions and potential oncogenic activity.[128] This could increase the possibility of using a targeted therapy even in combination with conventional chemotherapy.[127] Immunotherapy, even in combination with other therapies, could be another option for sarcomas with high MSI and/or high TMB.[129] *NTRK* fusions are rare genomic alterations that can be present in several sarcoma subtypes and have been identified as an agnostic biomarker for the treatment response with entrectinib and larotrectinib.[130,131]

Taken together, these results highlight the importance of incorporating comprehensive panels in the diagnosis and management of sarcoma, thus allowing a more precise differential diagnosis, treatment and the inclusion of patients in basket clinical trials.

Cancers of unknown primary

Cancers of unknown primary (CUP) account for approximately 3% - 5% of all tumors. CUPs can be divided into two main subgroups, with very different prognosis.[132] Approximately 85% of the diagnosed CUPs are included in the category of neoplasms with poor prognosis and short overall survival.[132,133] These are a group of heterogeneous metastatic tumors in which it is not possible to identify the site of origin and are the main focus of this subchapter. The treatment of these tumors is mainly based on chemotherapy regimens guided by histopathological features and likely site of origin. However, the results are not encouraging.[133]

How can NGS be used in cancers of unknown primary?

Recommendation: This type of tumor should be treated in centers with many patients/year. Since the only available therapy for CUPs with a poor prognosis is chemotherapy, the inclusion of these patients in Clinical Trials should be encouraged. Comprehensive NGS panels should be performed earlier in CUP, aimed at helping diagnose and direct therapy. Nevertheless, NGS testing must not delay the beginning of the approved therapy. The best strategy for each patient should be discussed on an individual basis and in multidisciplinary meetings.

NGS could represent a new option for these patients, providing insights into tumor biology, identifying potentially targetable genomic alterations aiming at personalizing the treatment of CUPs.[134] Comprehensive genomic profiling by NGS in CUP[133,135,136] has shown that although it was not possible to find a CUP-specific molecular signature,[136] almost all CUP samples have at least one clinically relevant genomic alteration that could influence personalized therapy.[135]

A recently published systematic review also showed that 85% of CUPs harbored at least one genomic alteration and 47.3% presented a potentially targetable alteration for approved/off-label/clinical trial available drugs.[134] The key mutated genes were *TP53, RAS, CDKN2A, MYC, ARID1A, PIK3CA, or BRAF*, which are not tissue-specific.[137-139]

One of the comprehensive CUP analyses also evaluated response to immune checkpoint blockade therapy. Mutations in 592 genes and 52 gene fusions in 389 cases of CUP were analyzed. TMB and MSI were calculated from the NGS results and showed that 11.8% of CUPs have high TMB and 1.8% MSI.[139] Thus, the multiplex testing approach calculated that 28% of CUPs harbored one or more predictive biomarkers (high-MSI, PD-L1, and/or high-TMB) to immune checkpoint blockade.[139]

Treatment decisions based on genomic alterations identified in CUP are only reported in case-studies since clinical trials are still ongoing.[140] A recently published non-randomized phase II clinical trial, conducted in Japan and involving 97 previously untreated patients with an unfavorable subset of CUP, showed that the gene expression profile and genomic alterations identified by NGS contributed to the

site-specific treatment of patients.[141]

The CUPISCO study is a phase II clinical trial for a CUP population that, through NGS techniques, will compare the efficacy and safety of targeted therapy or cancer immunotherapy versus platinum-based chemotherapy. The tested drugs include alectinib, vismodegib, ipatasertib, olaparib, erlotinib, bevacizumab, vemurafenib, cobimetinib, trastuzumab, pertuzumab, atezolizumab, carboplatin, paclitaxel and gemcitabine.[142]

One of the problems of tissue-agnostic therapy is the extrapolation of therapeutic actionability since the clinical activity of the mutations could differ between cancer tissues.[134,140] So far, putative primary sites have always been considered in CUP therapy.[134]

Nevertheless, the Cancer Genome Atlas demonstrated recently that the tissue of origin of a tumor might be less critical to prognosis and response to therapy than the identification of targetable mutations and optimal predictive biomarkers.[143,144]

FINAL CONSIDERATIONS

In which additional tumor types should NGS be applied as a diagnostic and therapy management tool or as a guide to clinical trials?

In these Portuguese consensus recommendations, lung cancer, sarcomas, and CUPs were included as the main types of solid tumors in which NGS must be performed for accurate tumor characterization and therapeutic decision. Nevertheless, there are other types of solid tumors, namely metastatic breast, and colorectal cancer, that may benefit from NGS use. According to the latest ESMO recommendations, NGS should be routinely used in patients with prostate cancer, ovarian cancers, and cholangiocarcinoma.[13] Patients with breast, colorectal, pancreatic and hepatocellular cancer should be included in clinical research for molecular screening programs proposing access to clinical trials with innovative agents.[13]

Comprehensive analysis of different types of cancers such as lung, colorectal, breast, ovarian, and sarcoma demonstrated that high-throughput techniques could identify an actionable mutation in a high percentage of cases, with clinical benefit in 25% of the patients.[145] Of the patients broadly tested by NGS, 37% have at least one clinically relevant mutation that could be targeted, cost-effectively, with either an off-label therapy or included in a clinical trial.[146] In the context of immuno-oncology, NGS is also an emerging technology through the identification of tumors with high MSI and TMB that will determine whether the patient is likely to respond to immunotherapy.[147,148]

The contribution of NGS to the deep understanding of genomic alterations that could occur in various tumor types has been studied in clinical trials, namely basket trials.[149] Basket trials include patients that harbor the same genomic alteration regardless of the histology.[131] These trials are of particular interest for patients with hard-to-treat tumors, which are commonly advanced tumors after multiple lines of therapy and rare malignancies.[150] Different basket trials have been designed and developed to detect genomic alterations that have a clinical benefit to patients with intractable cancers.[149,150] The results of different basket trials have demonstrated that molecular-targeted cancer therapy could benefit unmanageable cancers; nevertheless, there is a need to improve the selection of the molecular alterations.[151-174] In Portugal, there are two ongoing biomarker-driven clinical trials in solid tumors with FGF/FGFR aberrations[175] and *NTRK* Fusion-Positive Tumors.[176] The TAPISTRY trial, a phase II global multicentric study that evaluates the safety and efficacy of targeted therapies or immunotherapy in patients with an unresectable, locally advanced or metastatic solid tumor that harbor actionable genomic alteration or high TMB validated by NGS, is currently recruiting in Portugal.[177]

Finally, evaluation of patient outcomes showed that NGS testing could positively impact progression-free survival with manageable healthcare costs[178] and improved clinical outcomes in 33% of metastatic cancer patients with "hard to treat" disease.[153]

Thus, the benefit of NGS testing may impact the management of cancer patients, regardless of tumor type. Apart from the previously mentioned indications (non-squamous NSCLC, sarcoma, and CUP), NGS can be proposed for patients with metastatic disease, pending the discussion between the patient and the attending clinician of the expected benefits and the economic evaluation by the healthcare payer.[8]

CONCLUSION

NGS is a powerful technique that can identify predictive biomarkers for a targeted therapy that otherwise might not be considered. With this information, along with the expertise of multidisciplinary molecular tumor boards, clinicians will develop an optimal treatment plan for their patients. At this time, non-squamous NSCLC, sarcomas, and CUP are the main tumor types in which NGS should be used. However, in metastatic patients, NGS can be considered for all types of tumors where the standard of care has been exhausted and targeted therapy is still possible, especially if clinical trial participation is considered. NGS should also be considered if a new drug is available or there is a high clinical suspicion of the presence of a rare mutation. Furthermore, NGS data should be integrated with medical records and hospital information systems, allowing the creation of data repositories for clinical investigation. These approaches will allow for more patients to be treated with different therapeutic options.

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