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A call to action: molecular pathology in Brazil

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Abstract

Background: Adoption of molecular pathology in Brazil is currently very limited. Of note, there are no programs for training new molecular pathologists in the country; thus, documents compiling nationally applicable information on molecular pathology are few.

Methods: A selected panel of Brazilian experts in fields related to molecular pathology were provided with a series of relevant questions to address prior to the multi-day conference. Within this conference, each narrative was discussed and edited by the entire group, through numerous drafts and rounds of discussion until a consensus was achieved.

Results: The panel proposes specific and realistic recommendations for implementing molecular pathology in cancer care in Brazil. In creating these recommendations, the authors strived to address all barriers to the widespread use and impediments to access mentioned previously within this manuscript.

Conclusion: This manuscript provides a review of molecular pathology principles as well as the current state of molecular pathology in Brazil. Additionally, the panel proposes practical and actionable recommendations for the implementation of molecular pathology throughout the country in order to increase awareness of the importance molecular pathology in Brazil.

Keywords: Precision medicine, Molecular pathology, Brazil, cancer care, Molecular biology

Introduction

In recent years, many advances in oncology drug development and a deeper understanding of tumor biology and immunology have prompted further development of target drugs to specific tumor aberrations. In the new era of personalized cancer care, strategies are being developed and tailored for cancer detection and anti-tumor treatments associated with distinct clinical features, unique molecular profiles, and each individual patient's tumor microenvironment. Improved diagnostics and molecularly targeted drugs have revolutionized cancer treatment in the past decade. Rather than coalescing critical data for biomarker discovery, individualistic categories of "omic" data (e.g., genomic, proteomic, metabolomics) are heavily relied upon. Collectively, biomarker discovery and validation challenges, such as biomarker identification, prioritization, and integration, better reflect the complexity of the necessary data surrounding cancer treatment (Ferreira et al. 2016).

The objective of molecular tests in oncology is to detect structural and functional alterations in DNA, RNA, and proteins that will identify potential biomarkers. Whatever the alteration sought, the molecules' integrity is fundamental for determining a precise diagnosis, prognosis, and predicting response to specific therapies. DNA holds all gene sequences, which are transcribed into RNA and later translated into proteins that exercise cellular functions. Recognizing these alterations provides

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a more personalized treatment since many drugs can act on specific targets and molecular alterations (Lindeman et al. 2017). Pathologists are essential to the practice of oncology because they provide information for diagnostic test selection, performance, and interpretation, as well as assess the implications of results on care decisions. In addition to providing an accurate diagnosis based on morphological characteristics, pathologists should guarantee the integrity of these molecules during histological processing and, in turn, secure the result of quality molecular tests. It is important to note that the quality of a specimen can be affected at many stages of its journey and proper handling by all personnel involved (Lindeman et al. 2017).

The evolution of genetic molecular diagnosis has paved the way for the implementation of truly personalized medicine (Lindeman et al. 2017). Although the combination of molecular tests and their corresponding treatments have significant applications in oncology, their recognition and accessibility vary throughout the world. This paper attempts to provide an overview of basic principles of molecular biology and applications of molecular testing in pathology. Finally, this paper will analyze the current situation regarding access to molecular testing and therapies in the Brazilian health system, which includes a public health system (the Sistema Único de Saúde, SUS) and a private health system that work independently. Knowledge can help transform the clinical care of patients in places where molecular diagnostics are not yet sufficiently established. In this manuscript, example tumor types have been selected to create a list that is comprehensive for the basics of molecular pathologyin the country; these examples were chosen based on 1) availability of the drug matching the test, 2) availability and accessibility of the test, and 3) the common tumor types in Brazil.

Materials and methods

The Americas Health Foundation (AHF) identified clinicians and scientists with an academic or hospital affiliation who are experts in the field and who have published in the Brazilian pathology arena since 2014. As a result, AHF convened a six-member panel of clinical and scientific experts from Brazil. Great attention was paid to ensure a diverse group representing various disciplines related to molecular pathology. To better focus the discussion, AHF staff independently developed specific questions, addressing the salient issues on the subject, for the Panel to address.

Questions Provided to the Panel

1) What is the critical role of pre-analytics in the molecular analysis of patient biospecimens? Additionally, please explain the following:
a. How to handle surgical specimens properly;
b. The importance of formaline during fixation;
c. The critical role of sample quantity;
d. Less common situations, such as decalcification of bone tissues.
2) What are the main alterations in DNA, RNA, and proteins? What are the different types of molecular techniques? What are the advantages/disadvantages and utility for each technique?
3) Inside a molecular lab, what are the minimum requirements for molecular testing and reporting? How is quality control maintained?
4) Please give a brief overview of each of the following concepts:
a. Concept of biomarkers (i.e., the difference among predictive, prognostic, and diagnostic);
b. Biomarkers for immunotherapy;
c. Biomarkers for target therapy;
d. Concept of agnostic biomarkers (i.e., NTRK, MSI, etc.).
5) What drugs and biomarkers are currently available in Brazil? What drugs are available in the United States and Europe? Please note, this may be best explained through a table of main drugs and biomarkers.
6) What does access to molecular testing in Brazil look like? What are the current laws and regulations (i.e., reimbursement, reflex molecular testing, etc.)? Who has the ability to request a molecular test?

A written response to each question was initially drafted by a different member of the Panel. During the multi-day meeting of the Panel, each narrative was discussed and edited by the entire group, through numerous drafts and rounds of discussion until complete consensus was obtained. The objective of this article is to create a practical document addressing the adoption of modern technologies for molecular pathology in Brazil.

Search Strategy and Selection Criteria

Manuscripts referenced in this consensus paper were identified through searches of Pub Med and Embase with the search terms "molecular pathology", "pathology", "precision medicine", "personalized medicine", and "molecular biology" from November 2014 to November 2020. Articles were also identified through the bibliographies of the papers identified in the search as well as from sources of the authors' own files. Particular attention was paid to papers that reviewed or summarized the topic in question, or that were related to activities in the public health system of Brazil. The final reference list was generated on the basis of the relevance to the broad scope of this consensus document.

Principles of molecular biology

DNA contains all gene sequences which will be transcribed into RNA and later translated into proteins that will exercise cellular functions. The genetic alterations involved in neoplasm formation include abnormal protooncogene activation (leading to a gain of function status) or tumor suppressor gene inactivation (through loss of function). Recognizing these changes can lead to more personalized treatments since many drugs are able to act on specific targets and molecular changes. Tumor sample alterations can be 1) genetic or epigenetic and 2) somatic or germline. Genetic alterations occur within the DNA sequence, while epigenetic ones, also called epimutations, interfere with gene expression but do not affect DNA sequence.

Somatic alterations in the context of cancer are found only in tumor cells. Germline mutations are detectable within germ cells that can be passed on to the offspring, affecting all the body's cells.

The genetic alterations at the DNA level that may be detected and are relevant for patient care include the following: changes in the sequence of the genetic code such as single nucleotide variants (SNV), changes in the amount of genetic material-like amplifications and deletions (copy number variation [CNV]), and structural variants (SV). These mutations could be represented by substitutions, deletions, insertions, inversions, duplications, conversions, and deletions-insertions in the genome and may lead to different types of consequences within the protein level, such as missense, nonsense, or frameshift consequences (Fig. 1) (Lindeman et al. 2017). Silent and synonymous variants are alterations of a single base pair that in general do not result in disease repercussions (Le Tourneau et al. 2015). Other types of mutations are represented by large alterations such as translocation, inversion, amplification and deletion of gene or chromosomal regions. (Fig. 2). Of note, the clinical significance of all the mutations detected is not yet known.

Reports of the somatic variants in a molecular pathology laboratory should follow the Human Genome variation society (HGVS) recommendations and include the reference sequence, the alteration in the DNA level, and the predicted protein alteration using the 3 letters nomenclature for the amino acid (den Dunnen et al. 2016). Not all alterations in the DNA sequence or protein structure interfere with a particular gene function or represents a clinically relevant finding. The variants are interpreted regarding their relevance to gene function (based on databases) and in terms of clinical care and may be categorized by actionable, not actionable, or be variants of uncertain significance (VUS).

Primary techniques in molecular testing

Several techniques are available to detect genomic alterations in a tumor sample for clinical purposes in the molecular pathology diagnostic scenario. The techniques can be divided into those that involve a molecular (DNA/RNA) extraction and those that do not. Those involving a molecule extraction can be divided into non-sequencing and sequencing techniques. For both DNA and RNA extractions, the most used principles of purification are based on column filter or magnetic bead separation and are designed for manual, automated, or fully automated extractions (Kocjan et al. 2015). Table 1 compares these techniques in terms of turnaround time, type of alteration detected, coverage, and detection of other biomarkers.

Immunohistochemistry

Immunohistochemistry (IHC) is a technique based on antigen recognition in the tissue using chromogenic-labeled antibodies and retains a very important role in

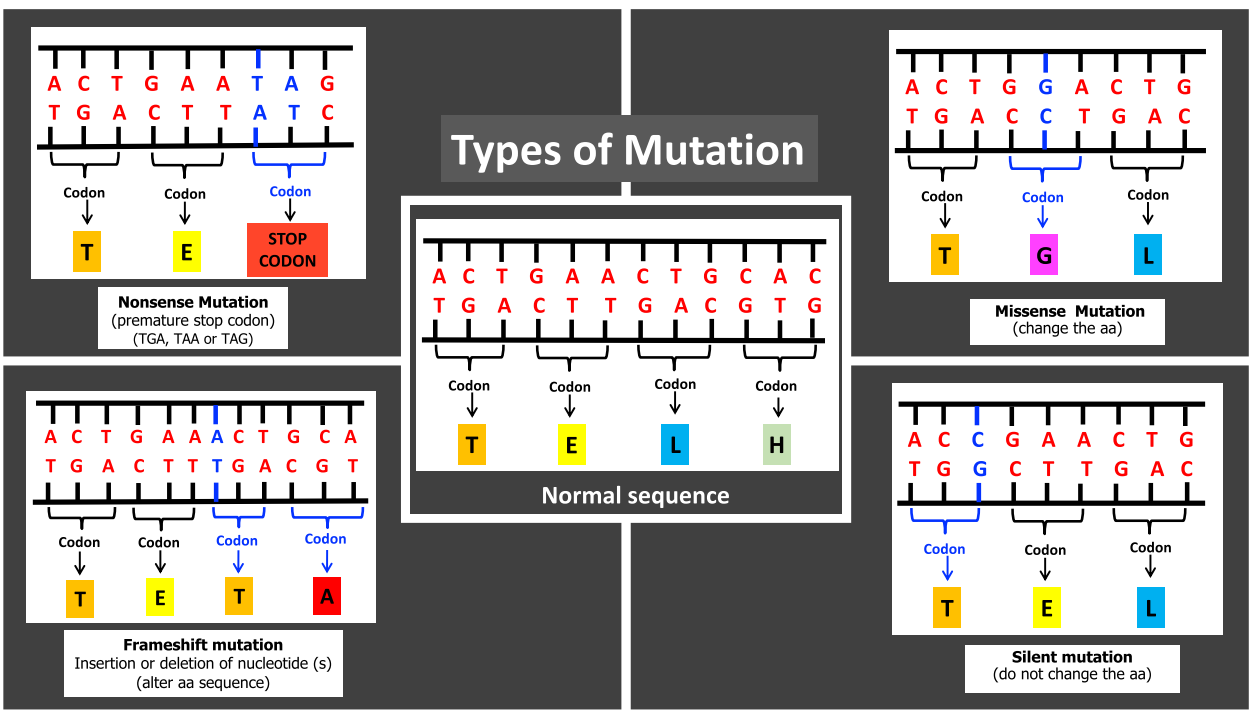

Fig. 1 Small DNA sequence variations and corresponding consequences. Legend: aa = amino acid

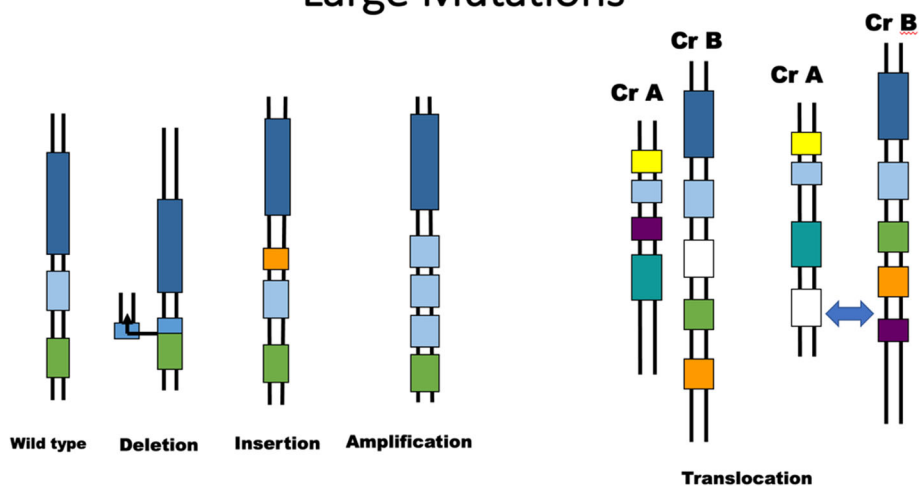

Fig. 2 Chromosomal variations

pathology. Despite advances in the molecular era, IHC is essential for many routine diagnostic workups by the pathologist and has a new role in molecular pathology. Using IHC, antibodies can identify genetic alterations by targeting proteins that reflect gene translocation, amplification, and mutational events. They can demonstrate a specific mutated gene product within the tissue and identify protein loss or overexpression caused by deleterious or activating mutations. In this way, IHC may be a cost-effective technique for identifying molecular alterations (Swanson 2015).

In situ hybridization

In-Situ hybridization (ISH) refers to the identification of genetic alterations and tissue morphology. The process consists of hybridizing fragments of DNA (probe) labelled either with fluorescence (FISH), chromogenic (CISH), or metallic (SISH) labels in the tissue or cytology specimen to identify the counterpart of that sequence. Interphase FISH is performed in the molecular pathology scenario and allows the detection of gene translocations, deletions and amplifications. The two main types of probes used in interphase FISH are centromeric (CEPS) and locus specific identifier (LSI). The former allows the detection and enumeration of specific chromosomes, and the latter is hybridized with the specific genes of interest, permitting copy number assessment and location of alterations. ISH used to identify amplifications and deletions are designed to look for a centromeric region and an allele specific region of the target gene,

Table 1 Comparison of different techniques used for detecting actionable alterations in molecular pathology

	SNV + Indel	CNV	SV	Tumor burden quantification (TMB)	MSI/MMR assessment	Hotspot (HS) alteration versus Whole gene evaluation (WG)	Morphology Correlation	Quantification of the finding	Turn Around time (estimated days)
In situ hybridization (ISH)	✖	✓	✓	✖	✖	HS	✓	✓	1d
Immunohistochemistry (IHC)	✓[a]	✓[a]	✓[a]	✖	✓	HS[a]	✓	✓	1d
Conventional PCR	✓	✖	✖	✖	✓[b]	HS	✖	✖	2d
Real Time PCR	✓	✓	✓	✖	✓	HS	✖	✓	2d
Digital PCR	✓	✓	✓	✖	✓	HS	✖	✓	2d
Sanger	✓	✖	✓	✖	✓[b]	WG	✖	✖	3d
Pyrosequencing	✓	✖	✖	✖	✖	HS	✖	✓	3d
Next Generation Sequencing (NGS)	✓	✓	✓	✓	✓	WG	✖	✓	5-10d

MMR mismatch repair system, *MSI* microsatellite instability
[a]IHC may aim proteins that reflect these alterations
[b]MSI by PCR runs in capillary electrophoresis the same equipment used in sanger sequencing

followed by a quantification assessment (Shakoori 2017; Cui et al. 2016; Levsky 2003).

Chromosomal translocations may be detected by break-apart or fusion probe ISH. Break-apart ISH consists of marking two physically close areas on the chromosome with two different colored probes. Translocation is detected when one pair of the signals is separated from the other. The fusion ISH is designed to identify translocation among two known partners and entails labelling each area of the chromosome with a different color probe. Translocation is detected when one set of fused signals is identified (Fig. 3) (Cheng et al. 2017).

Polymerase chain reaction

Polymerase chain reaction (PCR) is the most commonly used technique in molecular testing laboratories and is also involved in several other techniques. It consists of creating multiple artificial copies of a target DNA region flanked with a primer using the elements of cell replication, such as DNA polymerase and nucleotides. There are three general steps involved in a PCR. First, the denaturation of the DNA separates the double strand. Second, the primer anneals to its complementary bases, and third, the new DNA chain elongates. Each step occurs at a set temperature and is cycled multiples times, creating an exponentially high numbers of DNA copies (Netto et al. 2003).

There are several different types of PCR. A PCR performed with fluorescent dye may allow real-time amplification monitoring and quantification of the final product. This reaction refers to real-time PCR (rtPCR) or quantitative PCR (qPCR). Reverse Transcription PCR (RT-PCR) is used to convert RNA into a double strand complimentary DNA (cDNA) sequence, which allows the detection of alterations using RNA as the start material (Netto et al. 2003; Allen et al. 2008). The two main chemistries involved in qPCR are intercalate dye in the double helix and fluorophore-labeled oligonucleotides that contains a reporter and quencher (Navarro et al. 2015).

In digital PCR (dPCR), DNA pieces are portioned into individual reactions in which amplification takes place. Because the DNA is portioned, it is possible to measure the number of molecules in a set sample without having a standard, as is seen in other types of PCR. In droplet digital PCR (ddPCR), a water-oil emulsion is used to sub-portion of the PCR solution into many droplets. It is a very specific and sensitive technology that allows the detection of very low levels of mutations in tumor samples (Olmedillas-López et al. 2017).

Sequencing

Sequencing aims to determine the order of the nucleotide sequence in a set sample. Sanger sequencing, the traditional method for genetic sequencing, which is also known as the chain termination approach, uses a

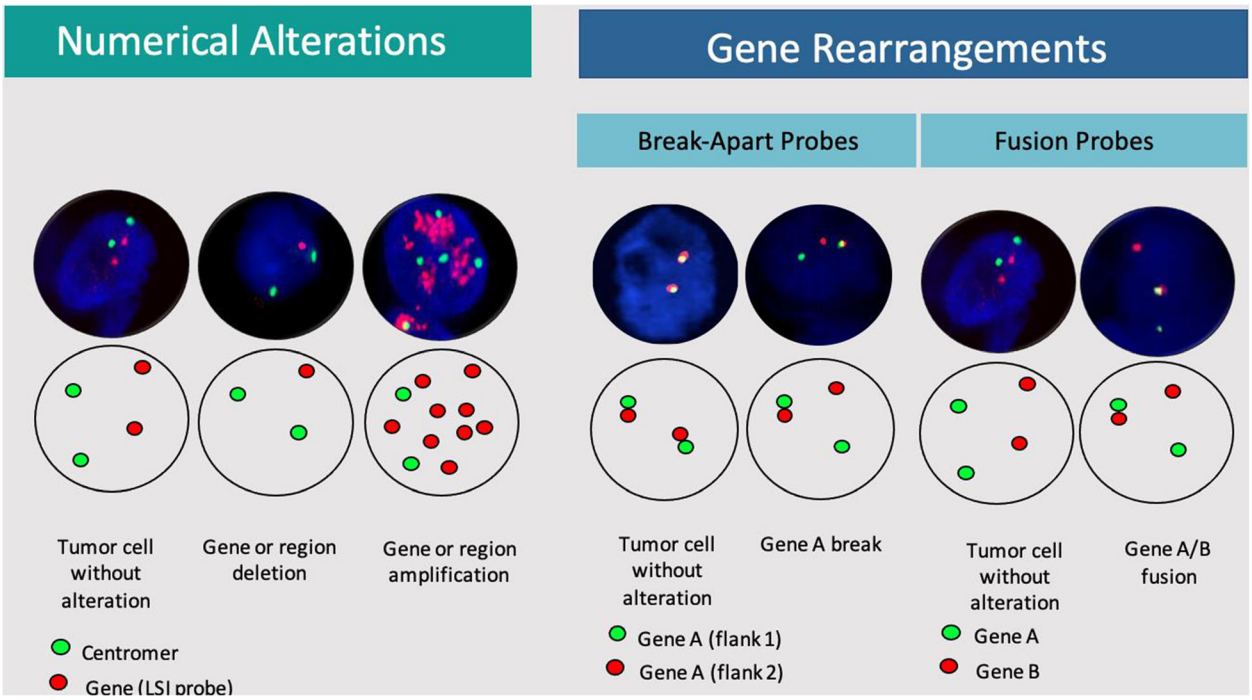

Fig. 3 Types of chromosome alterations detected by FISH

normal PCR combined with additional modified nucleotides in a fluorescent molecule. Sanger sequencing leads to the synthesis of different size DNA fragment and uses capillary electrophoresis to show the order of the nucleotides (Heather and Chain 2016).

Pyrosequencing, on the other hand, performs DNA sequencing based on the principle of synthesis. First, the targeted region is amplified and then sequenced by adding the deoxynucleoside triphosphate (dNTP), one nucleotide at a time. A nucleotide is incorporated in each complementary place in the DNA sample and, after a series of chemical reactions, releases a pyrophosphate that emits a visible light. This light is detected by the charge couple device camera and is recognized as a peak in the pyrography (Bluth and Bluth 2018).

Next-generation sequencing (NGS), also known as massive parallel sequencing or high throughput sequencing, may be used in oncology to detect gene alterations through whole genome, whole exome, or targeted sequencing panels. In the clinical setting, the use of target sequencing is more suitable than whole genome sequencing (WGS) or whole exome sequencing (WES) to generate relevant information for patient care, given the cost, turnaround time, personnel, and laboratory infrastructure required to perform the former two tests. NGS can be designed to cover hotspot mutations in specific genes or the entire coding or non-coding region of a set of genes. This methodology includes the following steps: sample preparation, library preparation, sequencing, and data analysis. Sample preparation for NGS is similar to most techniques that require a molecule extraction. The pathologist selects the specimen and makes a series of subsequent decisions such as testing the primary or metastatic tissue, assessing the pre- or post-treatment tissue, choosing the block in which the tumor area has the greatest tumor cellularity, and whether to perform microdissection for tumor enrichment (Jennings et al. 2017).

Library preparation is another step in the sequencing process and involves the preparation of the DNA and/or RNA for the sequencing step by fragmentation and addition of adapters. Enrichment of the targeted regions of the panel is also performed and may be based on hybrid capture (HC) or amplicon approaches. HC approach uses probes complementary to specific regions of interest, while the amplicon approach is based on the primers used for PCR amplification. The probes in HC are larger than the PCR primers, which allows some mismatches and better performance against allele dropout that might be observed with PCR approaches. The time to perform an amplicon library is shorter than the time to perform an HC (Jennings et al. 2017).

Sequencing involves a few other concepts. The Illumina platform and the ThermoFisher Ion system, currently the most prevalent instruments, are based on sequencing by synthesis detection. The Illumina platform uses fluorescent technology while the ThermoFisher Ion system uses ion semiconductor-based sequencing (Jennings et al. 2017). Both technologies have been shown to achieve comparable results (Fig. 4) (Misyura et al. 2016). Finally, four steps are necessary to generate data for the reports: read alignment, base calling, variant identification, and variant annotation.

Biomarkers

Evolution of biomarkers

Anatomic pathology has traditionally combined science and art. For more than 100 years, pathologists have used subjective aspects to establish a diagnosis, and most subjective aspects of these classifications are, in reality, morphological biomarkers. Pathologists pay particular attention to tumor classification. In this sense, the experience and training of pathologists are essential. For certain cancers, it is important to have biomolecular knowledge of tumor biomarkers and identify tumor alterations for a more accurate, targeted treatment. The concept of tumor biomarkers encompasses certain genes or proteins present in the tumor itself, which may indicate its degree of aggressiveness (prognostic markers) and predict the response to certain drugs (predictive markers) (Biomarkers in Cancer Immunotherapy n.d.; Mehta et al. 2010).

The basis of medical decisions in precision medicine is identifying a particular genetic alteration that serves the potential target of the molecular-designed drug. The search for the perfect drug (referred to as a “magic bullet” by Paul Ehrlich when he accepted the Nobel Prize for Medicine in 1908) starts with the alchemists, and the knowledge of the genome is one step closer to this objective. For example, when uropathologists classify acinar adenocarcinoma in the prostate, they automatically establish the Gleason score and cancer grade groups, which are the best prognostic biomarkers in this disease until now (Epstein et al. 2020).

The introduction of immunofluorescence in the early 1950s marked the beginning of the evolution of the state of the art. Immunofluorescence was the first objective tool used to establish the presence of a protein identified by an antibody and link it to a morphological diagnosis. This direct demonstration is an excellent method, but there are several technical difficulties, mainly because it requires the use of fresh tissues. The popularization of immunohistochemistry in 1980 and the subsequent years ushered in an era of new techniques, such as determining protein profiles by IHC. Biomarker use was extended to identify structural changes in DNA chromosomes and sequences and study RNA transcripts (Duraiyan et al. 2012; Bozorg-Ghalati et al. 2019).

The definition of biomarkers has evolved over time 2001; (EHC 155, 1993) n.d.). Currently, most researchers

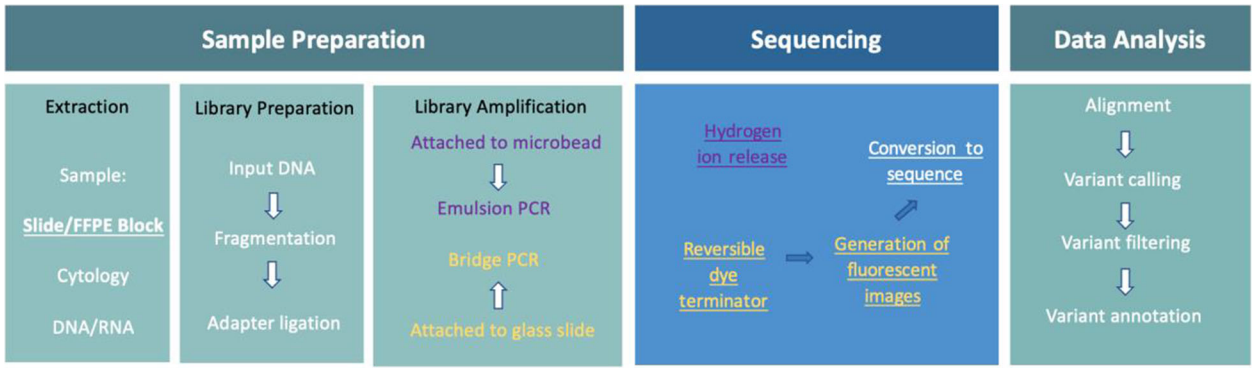

Fig. 4 Workflow from sample preparation to data analysis

agree that the most precise and comprehensive definition is provided by the joint initiative of the FDA and NIH, known as BEST (Biomarkers, EndpointS, and other Tools). This definition states that "A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. A biomarker is not an assessment of how an individual feels, functions, or survives" (FDA-NIH Biomarker Working Group 2016).

Biomarker development is critical (Strimbu and Tavel 2010; Nalejska et al. 2014; Selleck et al. 2017; Califf 2018). The use of biomarkers is diverse, including disease status monitoring, pharmacodynamic evaluation of an intervention, safety and adverse event evaluations of new drugs, risk susceptibility measurement in a determined population, diagnosing a disease or a specific subtype, identifying the probability of an endpoint event, and predicting the probability of success or failure of a given intervention. The primary biomarkers used in clinical practice can be seen in Fig. 5.

Biomarkers detected in anatomic pathology laboratories

The three main categories of biomarkers used in pathology are diagnostic, prognostic, and predictive and can be seen in Fig. 6. Diagnostic biomarkers are used to detect or confirm presence of a disease or condition of interest or to identify individuals with a subtype of the disease (Group F-NBW 2020). Prognostic biomarkers define a high-risk disease population, which can contribute to the development of new therapeutic strategies (FDA-NIH Biomarker Working Group 2016). These markers identify subpopulations that will behave differently within an already established disease, either with

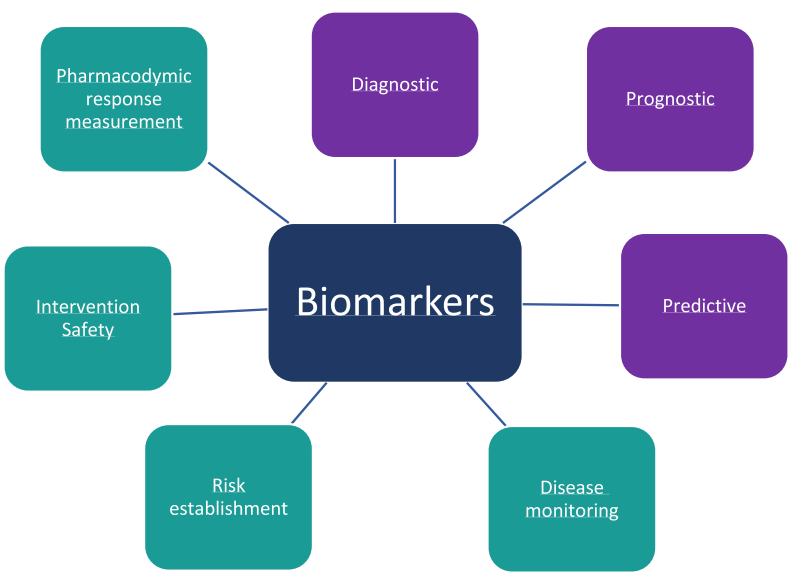

Fig. 5 Primary types of biomarkers used in clinical practice. *The three purple squares are discussed in the text; the four green are not

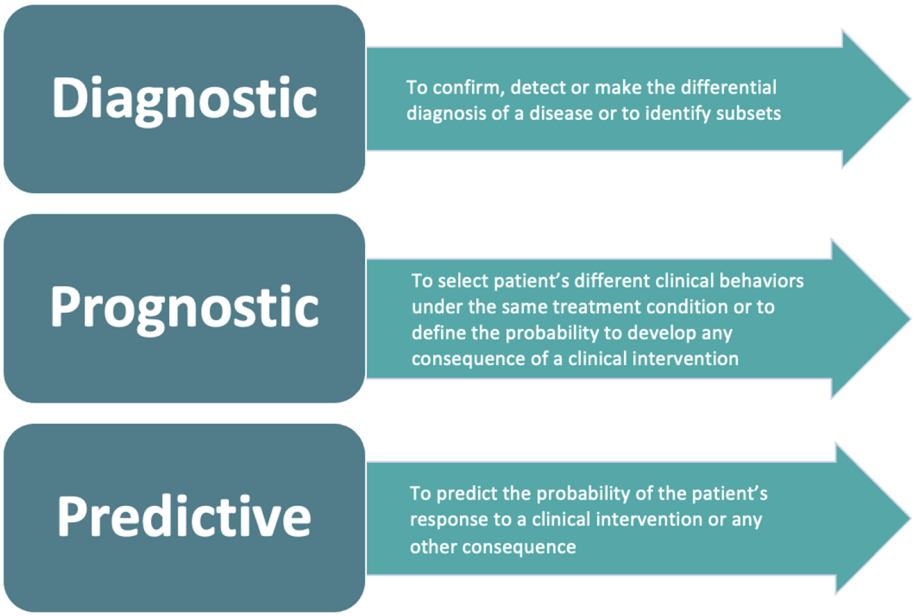

Fig. 6 Definition of diagnostic, prognostic, and predictive biomarkers

favorable or unfavorable disease evolution. Although there are many prognostic markers, their use in clinical practice is limited when the course of treatment is not affected. It is important to note that prognostic biomarkers should not be confused with "risk markers," which establish changes in health status and indicate the risk of developing a particular disease.

On the other hand, predictive biomarkers measure aspects of treatment response and identify a responder or high responder populations (FDA-NIH Biomarker Working Group 2016). Predictive biomarkers are used in clinical research to improve efficacy, reduce sample size, and increase the response to treatment, making trials more efficient. Some of these biomarkers, such as estrogen and HER2 receptors in breast cancer and CD20 in non-Hodgkin's lymphomas, have been used in the clinical setting for more than 20 years (Nicolini et al. 2018; Mohammed et al. 2019). The respective target drugs for these cancers were developed independently of the biomarkers.

A recent example is the *EGFR* mutation in lung adenocarcinomas. In the original article, the EGFR inhibitor was used without identifying which patients had the gene sequence alteration. Subsequently, samples were selected among the responders to understand that those who responded had mutations in exons 18–21 of the *EGFR* gene. Therefore, identifying and using the *EGFR* mutation as an efficient and excellent biomarker occurred after using the target drug in many patients. In contrast, the PD-L1 assay as a predictive biomarker was co-developed with the drug (Emancipator 2020).

Consequently, due to the selection made by the response to treatment, the biomarker is usually excellent and reproducible with a consolidated and widely used laboratory technique. Some clinical trials select a small number of patients based on a specific target indicated by a biomarker (Mandrekar and Sargent 2009). In other words, although the complete patient population may have the mutation, only some respond to treatment. In practice, the biomarker identifies the patients that have the mutation but does not define who will respond and who will not.

Drug approval depends on the therapeutic response and not on the efficiency of the biomarker. Moreover, a companion test may identify the correct patient, but it also has some discriminatory power because it does not necessarily identify the entire potentially targetable population. For example, the companion test for PIK3CA inhibitors in breast carcinoma covers about 70% of the actionable gene sequence changes present in the tumor, leaving part of the potential target population undetected (Martínez-Sáez et al. 2020).

Most recently, another major paradigm change is biomarker-driven trials that allow investigating patient heterogeneity on the basis of molecular profiling, which consequently introduces new opportunities and challenges. Master protocols are often classified into "basket trials," "umbrella trials," and "platform trials." Basket trials refer to designs in which a targeted therapy is evaluated on multiple diseases that have common molecular alterations. Umbrella trials, on the other hand, evaluate multiple targeted therapies for a single disease and

which are stratified into subgroups by molecular alteration. Both basket and umbrella trials employ a molecular screening protocol that allows either recruitment of different diseases with common molecular alterations or that discriminate a single disease into different molecular subtypes. Platform trials, also referred to as multi-arm, multi-stage (MAMS) design trials, evaluate several interventions against a common control group and can be perpetual. This design has pre-specified adaptation rules to allow dropping ineffective interventions and adding new interventions during the trial (Park et al. 2019).

Compared with the conventional paradigm, these trials require even more thorough planning and comprehensive evaluation on the overarching objectives (discovery or confirmatory). The trials also should evaluate the credential of biomarker's clinical utility, the choice of adaptive design and analysis plans, knowledge of cancer biology, existing data from preclinical and early clinical trials, prevalence of each subtype population, and the logistic readiness to conduct immortal clinical trials. Therefore, the success of biomarker-driven trials relies upon a closer collaboration among all involved in advancing cancer care, including physicians, clinical investigators, statisticians, sponsors, regulators, drug and assay developers, and patient advocates.

Biomarkers for targeted therapy

Monoclonal antibodies and small molecule drugs are the most prevalent targeted therapies 2019). The success of targeted therapy relies on finding molecular change; however, its presence is no guarantee that the treatment will work. The primary mechanisms of action, especially in tyrosine-kinase receptors, is blocking alteration activity and its signalization (Du and Lovly 2018). Although this is the primary goal of biomarkers for targeted therapy, these tests also may identify drug resistance or even establish toxicity (Dancey et al. 2010). Several drugs depend on companion tests, and many other clinical trials are under development to establish new biomarkers.

The uses of targeted therapy biomarkers vary and include IHC, in situ hybridization, comparative genomic hybridization, DNA and RNA sequencing, and transcriptional profiling. The method of choice may be linked to the drug's approval, forcing laboratories to use the method determined by the drug leaflet. Molecular profiling panels can cover many changes simultaneously, creating more therapeutic options (Malone et al. 2020). For example, within only a few years, lung adenocarcinoma expanded from one identified marker to several (Pennell et al. 2019). Ideally, changes in DNA such as *in EGFR (*Bethune et al. 2010*), ALK* (Shaw et al. 2009), *BRAF (*Brose et al. 2002*), KRAS* (Westcott and To 2013), *MET* (Drilon et al. 2017), *ROS1 (*Zinsky 2016*), ERBB2* (*HER*2) (Chuang et al. 2017), and *NTRK* genes (Haratake and Seto 2021), as well as PD-L1 (Pawelczyk et al. 2019) protein expression are explored before determining the most appropriate targeted therapy. When available, NGS is the most reasonable testing method for simultaneous detection of multiple alterations (Nagahashi et al. 2019). It is likely that other clear examples of the appropriate use of this method will emerge in the coming years.

Main clinical applications

The clinical applications of biomarkers used for solid tumors within the daily practice of molecular pathology, including colorectal cancer, lung cancer, breast cancer, and melanoma are discussed in greater detail in this section. Globally, access to diagnostic methods and treatments vary due to the differences in regulatory systems. In Brazil, not all biomarkers and associated treatments discussed in this text are approved or available.

Colorectal cancer

According to professional guidelines, colon and rectal cancer patients (CRC) benefit from testing the following biomarkers: *KRAS (*Edkins et al. 2006*), NRAS (*Irahara et al. 2010*)* and *BRAF* gene mutations (Barras et al. 2017), *HER2* amplification (Greally et al. 2018), *NTRK* gene fusions (Kheder and Hong 2018), and MSI/MMR status (National Comprehensive Cancer Network 2021a; National Comprehensive Cancer Network 2020).

Wild type *KRAS*, *NRAS* and *BRAF* CRC patients may benefit from anti-EGFR treatments such as cetuximab or panitumumab, either as single agents or in combination with chemotherapy in different advanced clinical scenarios and in agreement with prior treatments (Karapetis et al. 2008; Amado et al. 2008). *BRAF* mutated CRC with wild type *RAS* (*KRAS* and *NRAS*) may be eligible for a tyrosine kinase inhibitor (TKI) encorafenib treatment in combination with cetuximab or panitumumab (Tabernero et al. 2021). HER2-amplified CRC that is wild type for *RAS* and BRAF genes, may be eligible for trastuzumab treatment in combination with pertuzumab (Meric-Bernstam et al. 2019) or lapatinib (Tose et al. 2020) or to treatment with single agent fam-trastuzumab deruxtecan-nxki in many different clinical scenarios (Chau 2021). *NTRK* gene fusion positive CRC may benefit from TKIs, such as entrectinib (Siena et al. 2019) or larotrectinib (Kheder and Hong 2018), as single agent for subsequent therapy in progression or metastatic disease. MMR/MSI CRC patients may benefit from treatment with single agent nivolumab or in combination with ipilimumab as well as single agent pembrolizumab in specific clinical scenarios and

in agreement to prior treatments (Koncina et al. 2020; Overman et al. 2017).

Lung cancer

Proper treatment selection for non-small cell lung cancer (NSCLC) requires testing a wide range of biomarkers including *EGFR*, *BRAF* and *HER2* mutations; *ALK, RET, ROS1* and *NTRK* rearrangements, *MET* alterations (amplifications and *MET* exon 14 skipping mutation), PD-L1 expression and TMB assessment (Borghaei and Edelman 2020a). According to the NCCN guideline, identifying any of the previously listed molecular alterations may make a patient eligible for specific treatment, either as single agents or in combination with other drugs, depending on clinical and prior treatment settings. In lung cancer, specific clinical scenarios require re-testing for biomarker resistance in a tumor sample after treatment tumor progression (National Comprehensive Cancer Network 2021b).

Identifying an *EGFR* gene sensitizing mutations in NSCLC tumor tissue may make the patient eligible for treatments, such as TKIs, gefitinib (Maemondo et al. 2010), osimertinib (Ramalingam et al. 2020), or dacomitinib (Ramalingam et al. 2014); anti- *EGFR* antibody cetuximab in combination with afatinib (Goldberg et al. 2020); and EGFR TKI erlotinib hydrochloride in combination with bevacizumab or ramucirumab (Borghaei and Edelman 2020b; Nakagawa et al. 2019).

NSCLC harboring *ALK* gene rearrangements may be eligible for treatment with the following TKIs: alectinib (Peters et al. 2017), brigatinib (Hochmair et al. 2017), ceritinib (Shaw et al. 2017), crizotinib, or lorlatinib (Shaw et al. 2020). Patients whose tumors harbor *RET* rearrangements may be indicated to treat with the following TKIs: cabozantinib, vandetanib, pralsetinib (a RET-directed TKI), and selpercatinib (a kinase inhibitor or KI). Those with *ROS1* rearrangements may be indicated to be treated with the following TKIs: ceritinib (Lim et al. 2017), crizotinib (Shaw et al. 2019a), entrectinib (Dziadziuszko et al. 2021), or lorlatinib (Shaw et al. 2019b). Among the possible treatments for tumors harboring *MET* gene amplification or *MET* exon 14 skipping mutation are crizotinib and KI capmatinib (National Comprehensive Cancer Network 2021b).

Identifying *ERBB2* (*HER2*) mutations guides treatment with HER2 monoclonal antibodies and conjugates famtrastuzumab, deruxtecan-nxki, or ado-trastuzumab entansine (National Comprehensive Cancer Network 2021b).

BRAF V600E mutated NSCLC may be treated with vemurafenib or dabrafenib (either alone or in combination with trametinib). Entrectinib and larotrectinib are treatment options for NSCLC harboring NTRK1/2/3 gene fusions (Chu 2020).

PD-L1 IHC positivity is a biomarker to select NSCLC patients whose tumors likely benefit from atezolizumab either as single agent or in combination with bevacizumab and/or with chemotherapy. Nivolumab in combination with ipilimumab with or without chemotherapy, depending on tumor histology as well as pembrolizumab as single agent or in combination with chemotherapy. High TMB levels may be used to select patients for nivolumab treatment with or without ipilimumab (National Comprehensive Cancer Network 2021b; Borghaei and Edelman 2020b).

Because of the high number of the different biomarkers and treatments, NSCLC is a classic example where a comprehensive genomic profiling should be considered instead of multiple single tests. Cost, turnaround time, required tissue quantity, and technology availability are important factors to consider when dealing with lung tumor specimens (Borghaei and Edelman 2020b).

Breast cancer

According to professional guidelines, invasive breast cancer patients benefit from testing the following biomarkers: estrogen receptor (ER), progesterone receptor (PR), *HER2* (amplification), *PIK3CA* somatic gene mutation, *NTRK* gene fusion, and *BRCA1/2* germline mutation. PD-L1 IHC expression, MSI/MMR status, and TMB assessment are indicated for immunotherapy decision-making (National Comprehensive Cancer Network 2021c).

ER, PR, and HER2 are among the main biomarkers to be tested for invasive breast cancer management. These biomarkers guide a wide range of clinical treatment decisions either solo or in combination with other biomarkers findings. Treatment indications guided by hormonal and HER2 status include both single agents or combinations of different drug classes in a variety of clinical scenarios and prior treatments regimens. These include drugs such as HER2 monoclonal antibodies with or without conjugates (National Comprehensive Cancer Network 2021c).

PIK3CA somatic mutation in hormone receptor-positive (HR+) and HER2-negative invasive breast select patients eligible to treatment with the TKI alpelisib in combination with fulvestrant in specific clinical scenarios (Center for Drug Evaluation and Research 2019).

BRCA1/2 germline mutation information may guide recurrent or stage IV disease treatment with Olaparib or talazoparib for HER2-positive tumors and with Carboplatin or Cisplatin for triple negative tumors (Tung and Garber 2018).

NTRK gene fusion positive breast cancer may benefit from entrectinib or larotrectinib as a single agent for recurrent or stage IV disease that has no satisfactory alternative treatments or that has progressed despite treatment (Cocco et al. 2018).

PD-L1 IHC positivity in triple negative breast cancer (TNBC) is a biomarker to select patients with specific clinical presentation whose tumors likely benefit from atezolizumab or pembrolizumab treatments and possible drug combination scenarios with drugs such as carboplatin, gemcitabine, or paclitaxel (Cerbelli et al. 2017).

Breast cancers with MSI-H, mismatch repair deficient (dMMR), or high tumor mutational burden (TMB-H) (≥10 muts/mb) that have progressed after treatment and have no satisfactory alternative treatment options may benefit from pembrolizumab. Further, MSI-H is predictive of a better response to immunotherapy and improved OS in patients treated with pembrolizumab. TMB-H may also serve as a biomarker for decisions regarding bevacizumab treatment in combination with paclitaxel in specific clinical scenarios (Meehan et al. 2020).

Melanoma

Melanoma patients may benefit testing for the following biomarkers according to current guidelines: *BRAF, NRAS, KIT and NTRK* gene mutations (National Comprehensive Cancer Network 2021d). Approximately 50% of cutaneous melanoma harbor mutations in the codon 600 of the *BRAF* gene (Ascierto et al. 2012). *BRAF* mutated melanoma patients may benefit from the TKI vemurafenib in combination with either the MEKI cobimetinib or with atezolizumab. They may also benefit from treatment with the TKI dabrafenib as a single agent or in combination with the MEKI trametinib; or with the TKI encorefenib as a single agent or in combination with the MEKI binimetinib. All these indications depend on a variety of clinical scenarios and prior treatments (National Comprehensive Cancer Network 2021d).

Melanoma patients with an *NRAS* gene mutation may be eligible for treatment with the MEKI binimetinib as a single agent for metastatic or unresectable tumors that have progressed after prior immune checkpoint inhibitor therapy. Melanomas with *KIT* gene activating mutations may be treated with the TKI imatinib mesylate. Melanoma patients that harbor *NTRK* gene fusion may benefit from TKI treatment, such as entrectinib or larotrectonib in metastatic or unresectable disease as second-line or subsequent therapy for disease progression or after maximum clinical benefit from *BRAF* targeted therapy (National Comprehensive Cancer Network 2021d).

Biomarkers for immunotherapy

Immunotherapy is the main breakthrough in oncology in the past decade (How immunotherapy is used to treat cancer n.d.). The use of immune response checkpoint inhibitors, with CTLA-4 inhibitors in melanomas, followed by PD1/PD-L1 inhibitors, changed the landscape of cancer treatment (Haanen et al. 2020). There are now almost 70 different indications for immunotherapy (PD-1/PD-L1 Landscape n.d.).

Establishing biomarkers for immunotherapy is especially difficult because the checkpoint blockers' therapeutic action works in the interaction between tumor cells and the microenvironment (Gajewski et al. 2013). The search for reliable biomarkers is limited by an incomplete understanding of how immunotherapies modify the already complex immune response to cancer, as well as the contribution of immuno-editing to a dynamic and inducible tumor microenvironment and immune milieu. Further, these biomarkers vary according to the primary sites and tumors. Three tests usually are used as potential predictors for the indication of immunotherapy: microsatellite instability assessment (MSI), tumor mutation burden (TMB), immunohistochemical expression of PD-L1 (Luchini et al. 2019).

The evaluation of MSI, a condition of genetic hypermutability that results from impaired DNA mismatch repair, was the first agnostic indication for immunotherapy. In this scenario of agnostic use for this condition, the test is not predictive; rather, it is mandatory and discriminatory for immunotherapy. In other words, the agnostic indication depends on the identification of the molecular defect. IHC, PCR, or NGS are all suitable platforms, and the choice for which platform to select depends upon the availability and laboratory experience (Stinton et al. 2021).

TMB refers to the number of somatic mutations in a given tumor (Sholl et al. 2020). Interest in TMB started with the concept that tumors with many mutations would produce more neoantigens. The immune system must recognize these proteins and cause an inflammatory reaction promoting tumor control by cytotoxic action. Several clinical trials have demonstrated the relationship between TMB and response to checkpoint inhibitors. In June 2020, the FDA approved the use of pembrolizumab for tumors with a high TMB (Center for Drug Evaluation and Research 2020). This indication includes solid, unresectable, or metastatic tumors that have progressed with previous treatment lines and do not have satisfactory therapeutic alternatives.

Two main challenges to widespread use of TMB as a predictive biomarker are choosing the platform and the cut-off that should be used (Sholl et al. 2020). FDA approval was specific for the FoundationOneCDx assay as the platform of choice and a cut-off of 10 or more mutations/megabase (Foundation Medicine n.d.). Literature describes many other platforms with different coverages and high TMB definition limits, but these do not yet have FDA approval for use in this scenario. Using a specific platform (i.e., the FoundationOneCDx assay) limits

the test's application, and validation and correspondence between the different methods are essential. Likewise, the integration of TMB measurement and other aspects of the immune response require further studies. Therefore, although the use of TMB for immunotherapy is auspicious, its routine use in clinical practice is not widespread and faces several access barriers (Merino et al. 2020).

The use of IHC to determine PD-L1 expression is another factor in exploring biomarkers within immunotherapy. Tumor cells and inflammatory cells may contain a PD-L1 expression, which has been used as a companion test or complementary test in many immunotherapy indications. This marker has been widely described in literature and is far from being a perfect biomarker. Tumors that express PD-L1 generally respond better to immunotherapy; however, failures in predicting the response are relatively frequent (Acheampong et al. 2020; Bellesoeur et al. 2020).

Significant advantages of using PD-L1 expression with IHC are lower testing costs and widespread use (Davis and Patel 2019). However, different antibodies, with different types of interpretation, and different cut-off levels continue to present obstacles to expanded use. All these tests are associated with a specific drug and must follow the approved indication.

Currently, there are five available antibodies. The same antibody and methodology have different cut-off levels according to the tumor tested. The two most commonly used antibodies are 22C3 (Dako-Agilent) associated with pembrolizumab, and SP142 (Ventana-Roche), associated with the use of Atezolizumab (Tsimafeyeu et al. 2020). Figure 7 demonstrates the main characteristics of the available antibodies.

As previously indicated, the interpretation of results is also variable. There are currently several primary site indications for Pembrolizumab and the measurement of 22C3, and new applications are constantly being updated. Of these, the primary lung tumors are one of those that use the tumor positive score (TPS) with two cut-off levels of greater than or equal to 1% (i.e., low expression) or 50% or greater (i.e., high expression). In this situation, the calculation is quite simple: it is the ratio of the number of positive cells in the membrane to the total tumor cells expressed as a percentage. In the other situations, the use is the score known as CPS (combined positive score). This score contains the stained immune cells (lymphocytes and macrophages exclusively) and therefore also contains the numerator, which includes the tumor cells as well as the positive lymphocytes and macrophages. To calculate this score, the denominator is the number of tumor cells counted (Lantuejoul et al. 2020). This product is multiplied by 100 and expressed in absolute numbers, without decimals, on a scale of 0–100.

SP142's interpretation follows the same principles but with some essential differences. There are currently several primary site indications for using Atezolizumab and SP142 (VENTANA 2016). The most critical aspect for measuring positivity is that the results are expressed by the percentage of the tumor area occupied by positive

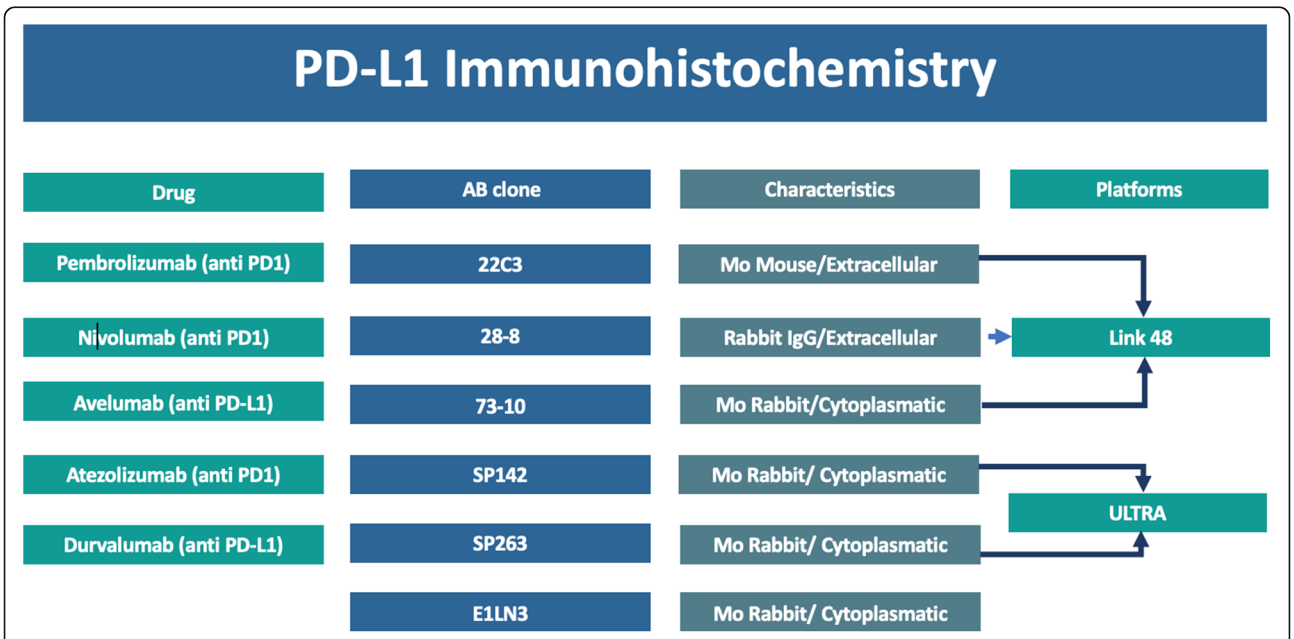

Fig. 7 Characteristics of the 6 PD-L1 biomarkers

cells, completely different from TPS or CPS calculations. Positive lung tumors are 50% or more of positive tumor cells or 10% or more of positive immune cells. When using SP142, all inflammatory cells (i.e., lymphocytes, macrophages, neutrophils, plasma cells) should be considered. Only inflammatory cells are considered regarding breast tumors and urothelial carcinomas, with cut-off levels of 1% or more and 5% or more, respectively (Lantuejoul et al. 2020). Table 2 summarizes the main differences among the primary sites, and antibody use.

Agnostic biomarkers

There are currently three agnostic biomarkers available for two targets. TMB and MSI/MRR are used for immunotherapy, and *NTRK* is used for targeted therapy. Recent years have seen a paradigm shift in research and regulatory approvals of cancer treatments. Pembrolizumab and larotrectinib the first approved agents for the treatment of solid tumors based on the presence of specific biomarkers rather than on tumor site, established the precedent of tumor-agnostic therapies. Pembrolizumab is a well-known anti-programmed death-1 (PD-1) T-cell receptor antibody. In 2015, the KEYNOTE-016 trial was the first study to show improved immune-related objective response and progression-free survival for colorectal cancer patients with deficient mismatch repair (dMMR) protein who were treated with pembrolizumab. In May 2017, the FDA approved pembrolizumab for the treatment of both adult and pediatric patients with unresectable or metastatic and microsatellite instability-high (MSI-H) or dMMR solid tumors (Marcus et al. 2019).

In November 2018, larotrectinib became the second drug to receive tumor-agnostic FDA approval for the treatment of adult and pediatric patients with solid tumors with *NTRK* gene fusions; this drug is now approved for use in Brazil. Larotrectinib is a tyrosine kinase inhibitor of the tropomyosin receptor kinase (TRK) proteins (i.e., TRKA, TRKB, TRKC), encoded for by the neurotrophic tyrosine receptor kinase genes (*NTRK1*, *NTRK2*, and *NTRK3*, respectively). In 2019, larotrectinib became the first tumor-agnostic cancer treatment approved by the European Union. Although the test is available in Brazil, only larotrectinib is currently an approved on-label drug to treat an agnostic tumor, and it is only available in the private system (Yoshino et al. 2020). Entrectinib is also approved by the FDA for adult and pediatric patients with solid tumors with NTRK gene fusions; however, this drug has not been approved in Brazil yet.

Additionally, another monoclonal antibody that targets the PD-1 receptor, nivolumab, had previously been approved by the FDA for the treatment of adults and children with MSI or dMMR metastatic CRC that had progressed following treatment with fluoropyrimidine, oxaliplatin, and irinotecan, as a single agent and subsequently in combination with ipilimumab (Center for Drug Evaluation and Research 2021).

Tumors that present *NTRK* fusions retaining the intact kinase domain are prone to respond to their inhibitors. and in this sense, follow the same logic of other target drugs. The frequency of *NTRK* fusions in tumors is roughly divided into three groups. The first group includes infantile fibrosarcoma, childhood gliomas, congenital mesoblastic nephroma, and secretory carcinomas of the salivary gland and its analogous in the breast and accounts for approximately 75% of these cases. The second group has a frequency of 5–75% and includes thyroid carcinoma, GIST, and melanomas. The third group includes all other types of tumors with a frequency of less than 5%. Of note, other alterations such as amplification and point mutations have not proven to respond to TRK inhibitors (Albert et al. 2019).

Fusions usually generate an altered protein that can be identified by IHC, a method highly sensitive but many times unspecific if the antibody is not specific to the rearrangement. However, the identification of the fusion is considered the gold standard and can be done by

Table 2 PD-L1 IHC test by tumor type in available for use in Brazil currently

Primary site	Indication	AB	Cells	Method	Cut-offs
Lung	Pembrolizumab	22C3	TC	TPS	≥1% e ≥ 50%
Lung	Atezolizumab	SP142	TC + IC	%IC/tu area	≥50% (TC) ≥10% (IC)
Stomach/EGJ	Pembrolizumab	22C3	TC + IC	CPS	≥1
Uterine Cervix	Pembrolizumab	22C3	TC + IC	CPS	≥1
Urotelial Carcinoma	Pembrolizumab	22C3	TC + IC	CPS	≥10
Urotelial Carcinoma	Atezolizumab	SP142	IC	%IC/tumor area	≥5%
Head & Neck - SCC	Pembrolizumab	22C3	TC + IC	CPS	≥1 e ≥ 20
Breast	Atezolizumab	SP142	IC	%IC/tumor area	≥1%
Esophagus	Pembrolizumab	22C3	TC + IC	CPS	≥10

EGJ esophagus gastric junction, *SCC* Squamous cell carcinoma

direct demonstration and visualization by in situ hybridizations or by DNA/RNA sequencing (Marchiò et al. 2019; Hsiao et al. 2019; Penault-Llorca et al. 2019).

IHC has a high sensitivity but less specificity (Solomon et al. 2020). The antibody for this test detects the C-terminal portion and is conserved in all NTRK proteins. The pattern of the reaction depends upon the gene involved in the fusion. The use of FISH, although highly specific, is recommended only in tumors with a high frequency of known fusions. Therefore, RNA sequencing is the preferred test, and the presence of an intact kinase domain is necessary to determine actionability. Confirmation is mandatory for IHC. Table 3 reviews the main biomarkers in solid tumors that are currently utilized in clinical practice.

Liquid biopsy

Currently, precision oncology uses tissue as the gold standard sample. However, tissue is not always accessible and sometimes provides limited information that does not account for the heterogeneity that can occur between different areas of the same tumor (spatial heterogeneity) or when comparing the primary and metastatic sites (temporal heterogeneity). Liquid biopsy relies on analyzing soluble factors in the blood, urine, or cerebrospinal fluid. This test has become a new means to obtain information about both the genomic composition of tumors and the tumor burden. The most important sources of biomarkers, in liquid biopsies, are the circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA). The presence of CTCs in peripheral blood after a thorough evaluation of prognostic biomarkers is associated with poor prognosis and high probability of metastatic disease. Compared with CTCs, ctDNA can provide a more accessible source for tumor genotyping because its level has been shown to be higher than that of CTCs (Neder et al. n.d.).

ctDNA is derived from tumor cells and likely released by apoptotic of necrotic cells or anoikis. CtDNA represents a fraction of the total cell-free DNA (cfDNA) derived from physiological tissue remodeling events. ctDNA alterations can be evaluated by a variety of techniques including allele-specific PCR, digital PCR, and NGS. Increased levels of ctDNA most commonly are associated with later stage disease or disease recurrence after treatment (Neder et al. n.d.).

Data have demonstrated that evaluating ctDNA can help identify genetic alterations for targeted therapy and mutations responsible for resistance when tissue biopsies are not feasible. Additionally, ctDNA potentially can be used for early detection of minimal residual disease, detection of early-stage cancer, and serial

Table 3 Main predictive biomarkers in solid tumors currently used in clinical practice

Biomarker	Alteration (main test used)	Associated Cancer
ALK	Rearrangements Specific mutations, rearrangements (sequencing, RT-PCR, IHC, ISH)	Lung, lymphoma, inflammatory myofibroblastic tumor (IMT)
BRAF	Specific mutations (sequencing, RT-PCR, IHC)	Melanoma, colorectal, NSCLC, thyroid, glioma
BRCA1/2	Specific mutations (sequencing, RT-PCR)	Breast, prostate, ovary, pancreas
EGFR	Specific mutations (sequencing, RT-PCR)	NSCLC
ER and PR	Protein expression (IHC)	Breast
FGFR2	Specific mutation/ rearrangement (sequencing, RT-PCR)	Biliary system, bladder
FGFR3	Specific mutation/ rearrangement (sequencing, RT-PCR)	Urothelial
HER2	Protein expression, gene amplification or mutation sequencing, (IHC/SH)	Breast, lung, colorectal, stomach, uterus
IDH1/IDH2	Specific mutations (sequencing, RT-PCR)	Biliary system
KIT	Specific mutations (sequencing, RT-PCR)	GIST, melanoma
MET	Specific mutation/ amplification (sequencing, RT-PCR/ISH	NSCLC, kidney
MSI/MMR	Multiple gene alterations/protein expression (Sequencing, RT-PCR/IHC)	Agnostic use
NTRK	Rearrangements (sequencing, RT-PCR, IHC, ISH)	Agnostic use
PD-1/PDL-1	Protein expression (IHC)	Several tumors
PDGFRA	Specific mutations (sequencing, RT-PCR)	GIST
PIK3CA	Specific mutations within PI3 (phosphoinositide kinase 3) (sequencing, RT-PCR)	Breast
RAS (KRAS/NRAS)	Specific mutations (sequencing, RT-PCR)	Colorectal
RET	Rearrangement (sequencing, RT-PCR)	NSCLC, thyroid
ROS1	Rearrangements (sequencing, RT-PCR, IHC, ISH)	NSCLC
TMB	Multiple gene alteration (NGS)	Agnostic use

ctDNA quantification to assess tumor burden (Neder et al. n.d.). However, major challenges to this expanded use include the lack of standardization, better understanding of the full potential and limitations of this technology, and insufficient knowledge of the tumor microenvironment and immunologic response to ctDNA released in the blood.

Best practices for molecular testing

The best approach for molecular diagnostics requires expert selection of the appropriate biological specimen and the most applicable test. During the process, it is necessary to evaluate test performance of the preanalytical and analytical phases in generating clinically useful patient reports. Compliance with operational and quality assurance requirements, which vary by geographical region, is crucial for the success of molecular laboratories.

Specimen handling

Molecular testing begins with biological material acquisition, in which specific procedures are optimized to achieve quality tissue preservation. These specifications can be found in the Brazilian Society of Pathology's Manual of Good Practices (Sociedade Brasileira de Patología 2020).

Prefixation The prefixation period is the time between tissue resection and processing. There are several environmental factors, such as transient anoxia and local pH changes, that may lead to molecular alterations during tissue excision (Srinivasan et al. 2002). However, because the variable factors of prefixation are determined mostly by the surgery's nature and are unmodifiable, the effort to minimize molecular alterations must begin after the tissue is removed. Ischemia begins immediately after tissue extraction and culminates in cell death through the destruction of molecules, with subsequent total degradation and tissue autolysis. Significant biochemical alterations occur just 10 min after anoxia (Kalogeris et al. 2012). Therefore, to reduce prefixation times, the fixation process must be carried out immediately.

Fixation Fixation involves proper training and a concerted effort from the entire surgical team. Tissue fixation has been used for more than a century and is the foundation of pathology. Although there are several chemical fixatives, the most widely accepted is neutral buffered formalin (NBF), a 10%-concentrated, aqueous solution that has several advantages, such as cost, quality, and long-term storage (Sociedade Brasileira de Patología 2020). Fixation is a vital part of the tissue process and cannot be oversimplified. All players involved in its process, such as surgeons, nurses, and pathologists, should be aware of its importance.

Proper solution preparation is an important part of the fixation process. Formaldehyde, the basis for NBF preparation, is a colorless gas that should be kept as an aqueous dilution of 37–40%. In anatomic pathology, a dilution of 1:10 produces the 10% NBF necessary for tissue fixation. The ideal preparation should include a buffered process to equalize the pH to 7.3 (Grizzle 2009). Although this practice is used often in pathology laboratories, it is important that administrators and those responsible for hospital pharmacy procurement in community hospitals that supply NBF understand the criticality of proper solution preparation.

Although widely used, formaldehyde-based fixation is not perfect and can harm molecules, because it has deleterious effects on the quality of RNA and DNA extracted from tissues. Cross-linking with aldehyde bridge formation can modify proteins and nucleotide sequences, leading to material degradation. Both DNA and RNA suffer fragmentation from fixation, the latter to a greater degree. RNA commonly is found in specimens ranging from 150 to 200 base pairs in length (Srinivasan et al. 2002; Howat and Wilson 2014; Chung et al. 2017). It is important to note that NBF is a toxic compound that should be handled with safety precautions (Helander 1994; Fox et al. 1985).

Sample quantity Tissue sample requirements are ever evolving in line with the development of new tests and drugs. The macromolecule quantity corresponds with the amount of viable tumor cells. Therefore, DNA/RNA quantity can be as critical as quality. In recent years, small biopsies have begun replacing excisional biopsies, perhaps because of the uptick in minimally invasive procedures, continuous introduction of new therapies, systemic treatment improvement, and neoadjuvant and radio therapy use. Although the material obtained is becoming smaller, the molecular information required for treatment is increasing, which can result in tissue exhaustion (Speiser 2012). Even when the material is sufficient for histological analysis and/or IHC studies, it is not necessarily sufficient for molecular testing. A sample must be larger than 25 mm^2 of viable tumor tissue to be sufficient for successful molecular testing (Gastman et al. 2020). Pathologists and treating physicians must be aware of this required measurement to minimize tissue wastage during sample preparation and avoid requesting unnecessary tests. Improved coordination between surgeons, interventional radiologists, oncologists, and pathologists is necessary to guarantee adequate tissue collection.

Pre-analytical parameters The primary goals of fixation are enabling the best possible performance in the morphological analysis, retained access to the antigenic epitopes, and as little as possible impact on the molecular integrity. Variables will change the performance, but the main factors that can be controlled are as follows:

- pH: Formaldehyde must be buffered, with a pH close to neutral (7.3). This level minimizes aggressions to the organic tissue.
- Temperature: Like any chemical bond, temperature directly affects its occurrence and integrity: the lower the temperature, the more time it takes for the reaction to occur, the higher the temperature, the faster it takes for the reaction it will occur. The recommended is room temperature (16–25 °C). Temperatures lower than 4 °C better preserves the acidic content of the nucleus (including RNA) with little loss of morphology. A temperature higher than 37 °C preserves the morphology but does so with protein denaturation and loss of molecular integrity (Bussolati et al. 2011; Chafin et al. 2013).
- Fixative penetration: The fixative only starts to act when it comes in contact with the surface of the region of interest. The penetrating power of formaldehyde is typically around 1 mm/h. In small samples, this action occurs immediately and is not a problem. Large samples take longer to reach the desired depth, at which point autolysis and prevent proper analysis are possible (Thavarajah et al. 2012). Therefore, such specimens must be pre-cleaved as soon as possible.
- Fixative volume: The fixative volume should be at least 10 times the sample volume, and the liquid should not be contaminated by other fluids that can interfere with the action of the fixative agent (Loomis and Alu 2016).
- Fixation duration: For best results with formalin, the recommended fixation period is between 6 h and 48 h (Loomis and Alu 2016). Fixing samples for less than 6 h may result in changes in the morphology, and fixation periods over 48 h may result in changes in antigenic and molecular preservation.

Special conditions of tissue preparation

Frozen material Although NBF is the most widely used fixative, frozen tissue is the best method to conserve DNA/RNA integrity for molecular studies. However, for histological purposes, frozen tissue morphology is not ideal because of disadvantages related to cost, transportation, and storage of the material, all of which prevent its widespread use (Srinivasan et al. 2002).

Decalcification of bone Demineralization is necessary in processing bone specimens. Decalcification solutions cause extensive DNA fragmentation. Therefore, strong mineral acids, such as nitric and hydrochloric acids, should be avoided when possible. Weak organic acids, such as acetic and formic acids, are available and can be less aggressive. Another alternative for decalcification is ethylenediamine tetraacetic acid (EDTA), which is mild, slow, and pH-dependent and can lead to better preservation of the molecules (Schrijver et al. 2016).

Cytology Cytology specimens may represent a high-quality source of genetic material to be used in molecular pathology. It may be obtained through fine needle aspiration (FNA) of palpable, ultrasound or CT guided mass, from endobronchial ultrasound (EBUS) and endoscopic ultrasound (US) biopsy and from transbronchial procedures. The aspirate material may be processed as direct smears, liquid based cytology, or both, with or without a cell block. It is good practice to perform rapid on-site evaluation (ROSE) of the material to ensure tissue is sufficient for a diagnostic workup. In general, direct smears are prepared, via air-drying or in an alcohol fixative (or both). The smears are then stained. The higher quality of cytology sample is caused by the lack of formalin fixation. To obtain DNA and RNA from direct smears, the tumor cells can be scrapped off the glass slide with the aid of a scalpel (Jain and Roy-Chowdhuri 2018; da Cunha et al. 2013).

Post-fixation

Occasionally, a molecular test is ordered years after the tumor biopsy or resection was obtained. In order for this molecular test to be successful and accurate, the material must be stored in proper conditions. The vast majority of the samples in pathology laboratories are embedded in paraffin after fixation (FFPE). The paraffin blocks must be stored in an environment protected from direct sunlight, without humidity, and in a temperature of 18–25 °C (Stumm et al. 2012). The Brazilian Society of Pathology recommends that paraffin blocks be preserved for at least 10 years and, when possible, even longer. When stored correctly, molecules, especially DNA, can be obtained from archived material for a molecular test. Figure 8 reviews the best practices for FFPE material fixation and storage.

Logistical specifications

Essential operational considerations for reliable diagnostic tests are documentation and reports, trained personnel, high-performing equipment, and information system management.

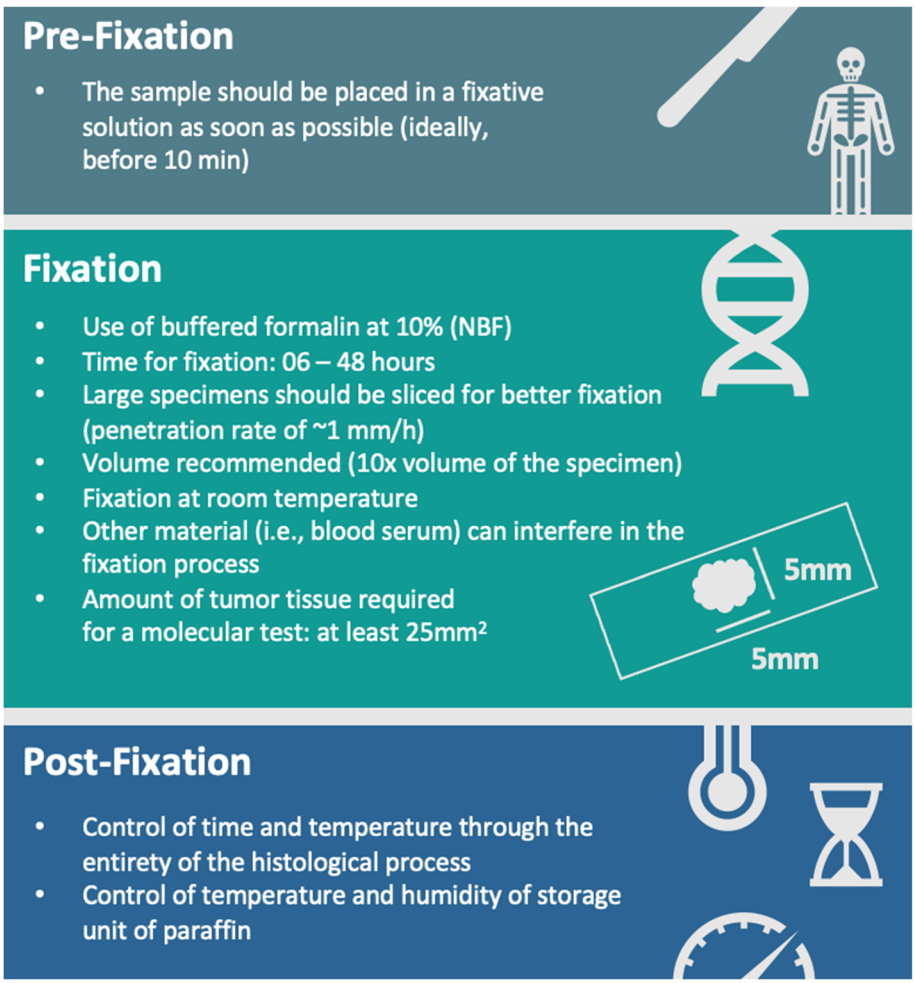

Fig. 8 Best practice for FFPE material fixation and storage for molecular testing

Documentation

Molecular test request orders, also known as the requisition forms, should require: 1) patient information, 2) ordering physician's name, 3) sample type, 4) time of collection 5) test to be performed and 6) any relevant information regarding patients' previous treatment or prior molecular testing, It may be important to consider relevant family history. Genetic testing requires informed consent of the patient or caretaker because the information obtained through this test may have risk implications for other family members.

Reports

Overall, the kinds of information that should be included in reports of molecular diagnostics are: 1) information about the tested sample (including sample type, sample identification and diagnosis, tumor purity and if any enrichment method was performed, 2) details of the methods (e.g., specific technique), 3) evaluated target, 4) findings, including list of relevant detected genomic alterations following the previously stated nomenclature guidelines and their corresponding variant allele frequency (VAF) and 5) interpretation (which should address the literature to support the conclusion). These also reports should inform the diagnostic sensitivity and specificity of the test. Limits of detection (LOD) should be included for oncology tests. Reportable and reference range may be required for specific tests. Reports must be released and communicated in a manner that maintains patient confidentiality.

Personnel

Experienced and trained personnel are of utmost importance for taking and delivering reliable and accurate tests. Specialty programs and certifications can help to meet the needs for qualified personnel. The laboratory director or authorized signatory must have experience in the field.

High-performing equipment

All platforms must be calibrated by appropriate accredited service agencies. Information, such as manufacturer's name, model, serial number, and contact information of the supplier, as well as the schedule for preventive maintenance should be on display on the instrument.

Information system management

The laboratory information management system (LIMS) is an important tool for managing samples, automating workflows, integrating equipment, and retrieving of information. The information must be secure during the entire process and comply with national regulations regarding data protection.

Quality assurance

Laboratories must have policies and procedures written to specify each step of the process and must include key indicators for monitoring the entire process. Nevertheless, ethical considerations in the operation of a molecular diagnostic laboratory are required for better patient service. The requirements that must be addressed are discussed in this section and summarized in Fig. 9.

Assay validation and assay verification

Before implementing a molecular test, the laboratory director must be aware of the regulatory status of the specific assay. Laboratory validation is required for a laboratory-developed test (LDT) or a test approved by the regulators and modified by the laboratory. Assay verification, on the other hand, should be performed for an unmodified approved assay. Laboratories should identify the test's specific use or application and demonstrate overwhelming evidence the test will perform consistently and as expected. Assay limitations are important to understand. For a LDT or modified approved assay, it is essential to determine analytic sensitivity, analytic specificity, precision, accuracy, reportable range, and reference range, along with any additional performance features. Assay verification of an approved test generally requires smaller studies with fewer samples than those required to validate an LDT or a modified approved test. This assay verification is necessary because the performance characteristics of the approved test have been established. Unmodified approved tests require evaluation of precision, accuracy, reportable range, and reference range (Jennings et al. 2009; Halling et al. 2012).

Assay controls

Assay control specimens that test preanalytical, analytical and postanalytical steps and are used with all patient samples are essential to ensure the best quality in the laboratory process. For most molecular assays, positive and negative control samples should be taken to guarantee that the findings are accurate.

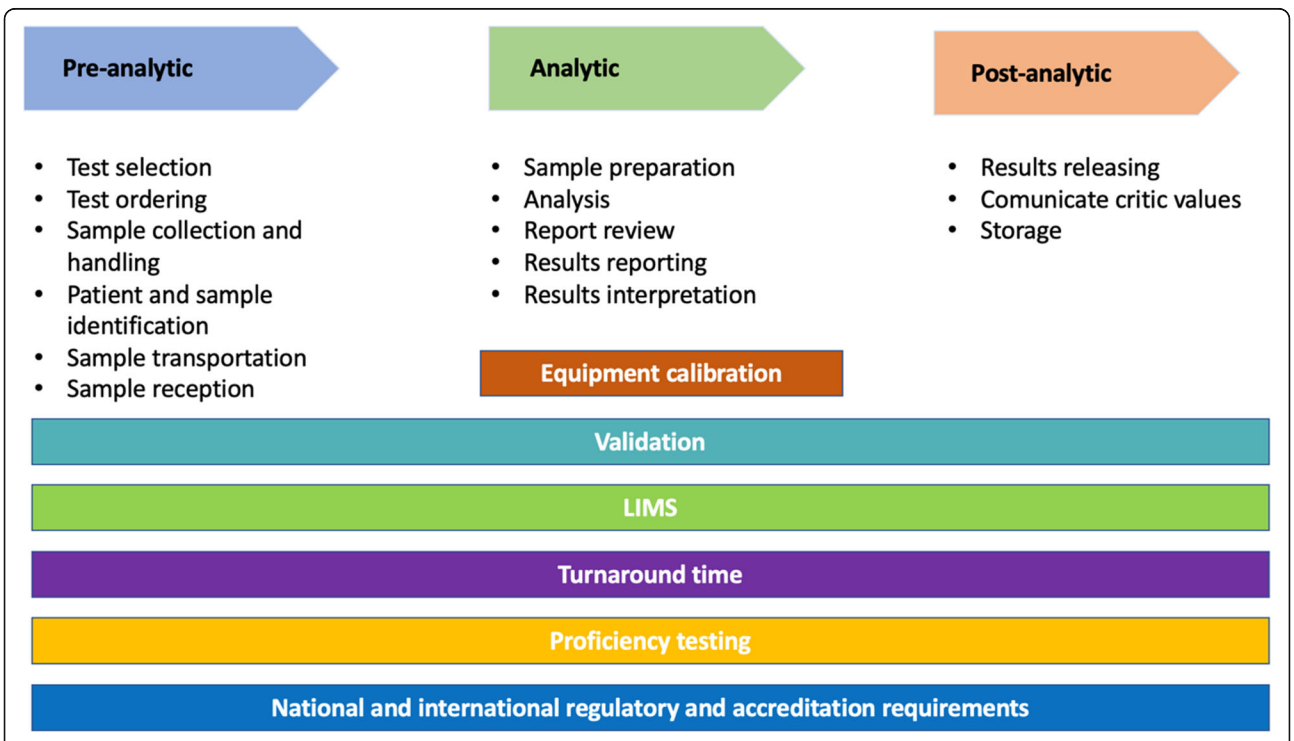

Fig. 9 Requirements for quality assurance, divided into the three analytic phases

Proficiency testing (PT)

Laboratories must be enrolled in a PT program and perform the evaluation for the tests at least semiannually to ensure optimal preanalytical, analytical, and postanalytical laboratory performance. If a particular test performed in the laboratory is not part of a PT program, there are some acceptable alternatives, such as sample exchange with other laboratories that perform the same test, split samples, or analysis with another method. A PT or alternative procedure should cover the entire testing process, sample preparation and analysis, and interpretation of the results, if applicable. Any challenges encountered must be documented, and solutions must be pursued.

Integrated diagnosis

In molecular oncology, it is important that diagnostic results be analyzed with the results of other tests, such as histo-cytopathology, IHC, flow cytometry, or cytogenetic assessment to reach a diagnosis. This collective analysis is referred to as integrated diagnosis.

Evaluation of turnaround time (TAT)

Appropriate turnaround times are critical for an efficient routine and to bring clarity for patients. TATs vary by test type and clinical application.

Standard operating procedure (SOP)

An SOP is a set of step-by-step instructions intended to help workers carry out routine operations; it is a tool that laboratory personnel use for safe and efficient guidance through a specific procedure. SOPs aim to achieve efficiency, quality output, and performance consistency, as well as to reduce miscommunication and improve compliance with industry regulations. The primary goal of a SOP is an accurate diagnosis, and training laboratory personnel in all aspects of these procedures is crucial to this goal. Appropriate personnel, including two supervisors, must review SOPs periodically and revise them as needed. The laboratory director must then sign off on each SOP. Methods, reagents, instruments, instrument software, and versions must be documented (Hawkins 2013).

Quality improvement

Monitoring indicators are needed to track trends over time to help identify problems in specific areas and assess where improvements are necessary and possible.

Accreditation

National and international accreditations help laboratories to establish and maintain a total quality management system. Accreditation improves the effectiveness of molecular diagnostic laboratories and ensures the best possible clinical care by using processes, methods, and technology that are consistent with established best practices (Accreditation Checklists n.d.; SBPC/ML|PALC publica Norma para Biologia Molecular n.d.).

Ethical considerations

Two main ethical considerations must be made when dealing with molecular diagnostics. First, laboratories must ensure the integrity of the results by keeping the highest standards throughout the entirety of the pathology process. This process includes documenting each person who handled the sample, the date and time it was collected or transferred, and the purpose for the transfer. Every effort must be made to minimize areas prone to human error (Cocks 2016; Ducatman et al. 2020). Second, confidentiality and proper disclosure must be prioritized because of the sensitive nature of the information contained in molecular diagnostic reports.

Communicating reports and molecular tumor boards

Molecular tumor boards are key platforms for discussing and communicating report findings. Such boards can guide decision-making in the clinical oncology practice and increase quality of care. This approach, based on standardized and evidence-based parameters, strengthens patient-specific therapeutic strategies through the identification of target alterations. Additionally, a tumor board composed of personnel trained in the pathologic, clinical, and ethical issues related to molecular testing creates a better approach to the application of the technology, data interpretation, clinical integration, and various ethical implications (Rolfo et al. 2018; Stoeklé et al. 2018).

Increasing access to molecular pathology in the Brazilian health system

A growing understanding of tumor molecular pathology combined with a surge of new drugs and associated diagnostic technologies has translated into improved survival rates for cancer patients. In addition to the technical considerations and complexities inherent to molecular pathology, achieving the potential that precision medicine has to offer will require overcoming hurdles within Brazil's healthcare system.

Expert interpretation is essential to inform clinical decisions in the complex scenario of a health system that lacks resources. This complexity continues to increase given the substantial constraints free public healthcare and the quality issues within the provision of health services. Pathologists have the opportunity to assume an important role and provide leadership including utilization management, precision medicine, reducing diagnostic errors, and improving healthcare outcomes.

Brazil has a public health system (Sistema Único de Saúde, SUS) and a private health system that work independently of the public system. Approximately 80% of the 200 million Brazilians are covered exclusively by SUS (Castro et al. 2019). This system has its own health technology assessment (HTA) body, the CONITEC (Comissão Nacional de Incorporação de Tecnologia), which is responsible for appraising approved drugs and other health technologies. CONITEC also provides guidance to the Ministry of Health and is responsible for incorporating selected recommendations into SUS. SUS's payment model is a fixed budget based on the disease. For example, if a patient with breast cancer receives first-line treatment, the government pays a predetermined amount and leaves decisions regarding the appropriate medical protocol to the health care provider, who works within the value of the reimbursement (Ferreira et al. 2016). Given the system's payment structure, access to innovative technology, high-cost medications, and limited procedures, it generally remains many years behind that seen in many developed countries.

Meanwhile, for the remaining 20% of the Brazilian population who can afford a private health care system, the pathway is different. Private health insurance is voluntary and supplementary to SUS. The National Agency of Supplementary Health (Agência Nacional de Saúde Suplementar, ANS) regulates the private system and periodically publishes a list of mandatory coverage procedures. Private health plans offer healthcare services through their own facilities or accredited healthcare organizations. Unlike SUS, private insurance can reimburse enrollees for purchased healthcare services. The private system is financed predominantly on the fee-for-service model, with remuneration to providers based on a published list of prices. Supplier–provider discounts are a significant financial component within this operation and therefore generate extensive criticism because this structure does not optimize resources. Therefore, even with the private health care system, there are significant barriers approving, adopting, and accessing new technology (Ferreira et al. 2016).

In both the public and private systems, molecular tests are not coded administratively. Although such coding may seem like a barrier relatively simply to overcome, pathology laboratories cannot translate test costs to the paying source because there are no specific codes (Ministry of Health Care n.d.). Furthermore, the disconnect between drug and companion test approval in Brazil creates a contradictory scenario in clinical practice. For instance, targeted drugs may be incorporated, but companion and complementary tests that support the use of these medications are not reimbursed and therefore are not broadly implemented.

Currently in Brazil, molecular tests can be accessed through sponsored pharmaceutical platforms and programs, making them available to a large proportion of the population. This well-intentioned but ill-conceived effort has given rise to a significantly complex issue. If these programs were interrupted, the test would not be accessible to the majority of the country's population—even when the test is commercially available. However, insurance companies favor this system because the burden of cost does not fall on them, thereby removing any pressure to recognize the tests and apply coverage strategies. Finally, testing through pharma-assisted programs is centralized and hinders the development of other pathology laboratories.

A reflex test is a diagnostic procedure that must be performed automatically under certain conditions. For example, a reflex test would be required if a female patient in the third year of follow-up treatment for breast cancer presents with a liver nodule. A biopsy followed by a diagnosis of poorly differentiated carcinoma would automatically trigger an IHC procedure to confirm the presence of metastatic breast cancer. At the same time, the test would check her hormone receptor (HR) status and HER2 expression. Confirmation of metastasis of breast cancer with HR+/HER2-would, in turn, automatically trigger another reflex test for *PIK3CA* mutation status (Tchrakian et al. 2016). With the private system, this potentially life-saving test cannot be carried out reflexively because patient consent is necessary given the likelihood of incurring out-of-pocket costs. Within the SUS, the laboratory is free to carry out SUS-approved tests. However, it usually only covers a fraction of the actual cost that the laboratory must pay to perform the test, which discourages implementation of quality testing.

Education and training in molecular pathology

Awareness of biomarker application and molecular pathology principles has become essential for practicing standard pathology. The pathologist's well-defined and crucial role in a molecular laboratory includes selecting the proper material for analysis and ensuring all the quality aspects of the test indications, sample utilization, analysis, and result interpretation. Additionally, a pathologist in a general anatomic pathology laboratory also has a big role in molecular pathology and is responsible for ensuring good quality material for future biomarker testing, properly handling, and optimizing the use of the tissue, precisely communicating with multidisciplinary teams to prioritize patient care, and selecting material for centralized tests. In this way, training in molecular pathology should be an urgent priority in the pathology residency training program, but many obstacles exist.

The pathology residency in Brazil is a 3-year training period during which the resident is responsible for demonstrating acquiring skills in many areas (e.g., gross and microscopic examination, intraoperative frozen sections, immunohistochemistry, autopsy, cytopathology, and laboratory management). Establishing molecular pathology fellowships for general pathologists to pursue after their initial training would create local training opportunities and increase accessibility to the field. Experts should discuss creating guidelines for the fellowship establishing minimal requirements, duration, infrastructure, centers, and trained faculty to make the best way to develop expertise in the molecular pathology field. Another limitation regards infrastructure and trained personnel. In Brazil, there are not many molecular pathology laboratories established in academic centers. This obstacle may be mitigated if residencies regularly discussed molecular pathology concepts regarding diagnosis, prognosis, and predictive information of the diseases being studied. Further involvement in molecular tumor boards or multidisciplinary discussions in the hospital setting would be beneficial for residency training. Continuing medical education, promoted by academic and professional societies, also may enhance widespread knowledge of molecular pathology (de Macedo et al. 2014). The complexities of a two-tiered healthcare delivery system are one of the main shortcomings for effectively implementing new fields of medicine (Castro et al. 2019; Macinko and Harris 2015; Araújo et al. 2011). Despite these challenges, Brazil has the potential to emerge as a model for achieving the opportunities offered by advances in oncology (Santos et al. 2019).

Conclusion

Despite the important and intense discussions around molecular pathology, physicians' experiences in resource-constrained situations appear to be more complex than the literature on healthcare rationing assumes. Molecular testing can be quite expensive in a resource-constrained health system; however, identification and treatment that possess a targeted genetic mutation and indicate which patients are likely to respond offer a substantial benefit. With molecular testing, the targeted therapeutic approach is used effectively, compared with the futile and expensive care that may result in unnecessary adverse events. At the same time, resource scarcity and the demand to produce more with less is an ever-present reality.

Precision medicine is an integral part of cancer care, and therefore, quality precision medicine should be encouraged wherever possible. The adoption of innovative technologies and techniques found in molecular pathology must be tailored to national realities and the associated constraints unique to Brazil. In addition to indicating therapies, molecular pathology can provide an undeniable benefit to those diagnosed with cancer in Brazil. International guidelines are increasingly recommending molecular pathology techniques; therefore, the stakeholders within the Brazilian healthcare system must understand that these techniques enable high quality and improved cancer care and that, subsequently, a shift is necessary to accommodate them. Accordingly, all actors must be aware of these new technologies and how to properly execute their role in molecular pathology, as outlined in Table 4. Recommendations on how to address the primary technical and access barriers follow.

Recommendations

Implement multidisciplinary care

✓ Promote multidisciplinary teams with all specialists involved in cancer care

✓ Create molecular tumor boards that include surgeons, oncologists, pathologists, geneticists, molecular biologists, and bioinformatic specialists, among other supporting experts to integrate specialties and ensure a comprehensive approach to diagnosis and management decisions

✓ Increase awareness of the importance of specimen handling and processing and the role of all players involved (e.g., surgeons, nurses, pathologists)

✓ Improve coordination among surgeons, interventional radiologists, oncologists, and pathologists to guarantee adequate tissue collection

✓ Understand, be aware of, and adhere to the clinical application of biomarkers in oncology.

Establish quality assurance

✓ Create quality standards to regulate molecular pathology testing at all levels and standardize reports

✓ Promote quality control programs for pathology laboratories and incorporate regulations specific to molecular pathology

✓ Thoroughly understand and be aware of the techniques used to detect actionable genomic alterations in the molecular pathology diagnostic scenario including IHC, ISH, PCR, and sequencing. These can be found in Table 1.

✓ Minimize tissue waste during sample preparation and avoid requesting unnecessary tests

✓ Uphold the highest standards of quality for molecular testing by adhering to the processes established for each method and procedure

Promote education on molecular pathology

✓ Address the shortage of cancer care specialists by creating national training programs specific for molecular pathology and incentivize specialists to go into the field

Table 4 Roles and responsibilities of personnel throughout the molecular testing pathway

Pathway of molecular pathology	Responsibility	Quality Assurance
Biopsy request	Referring physician	
Proper sample collection	Radiologist, surgeon, endoscopist, pathologist	
Sample handling and transport to the lab	Operating room staff/multidisciplinary team	
Specimen processing in pathology lab	Pathology lab multidisciplinary team	
Original diagnostic report	Pathologist	
Requisition form for molecular test and indication	Oncologist	
Sample preparation and processing	Molecular pathology multidisciplinary staff	
Comprehensive molecular report	Molecular pathologist	
Discussion with molecular tumor board	Multidisciplinary team	

✓ Train general pathologists in the principles of molecular pathology and best practices of specimen handling

✓ Incorporate basic molecular pathology training into all specialties involved in oncology care

✓ Develop peer-learning programs for improved knowledge transfer

Expand access to molecular testing

✓ Adapt national guidelines to the international standard of care so that they include recommendations on the use and clinical application of molecular tests and biomarkers in oncology

✓ Align all stakeholders, including medical societies, payers, and regulators, adopt precision medicine strategies, such as molecular pathology

✓ Create health policy that incorporates innovative technology in oncology, such as molecular pathology techniques and therapies, and ensure opportune and equitable access for the entire population

✓ Approve targeted therapies along with the corresponding molecular test concurrently to enable early initiation of treatment

✓ Ensure coherence in the therapies approved and reimbursed within the Brazilian health system with the recommendations on molecular testing and corresponding target therapies found in clinical practice guidelines

✓ Create specific codes for molecular tests to be ordered and charged appropriately by the health system

Abbreviations

AA: Amino acid; AHF: Americas Health Foundation; ALK: Anaplastic lymphoma kinase; ANS: Agência Nacional de Saúde Suplementar / National Agency of Supplementary Health; BEST: Biomarkers, EndpointS, and other Tools; BRAF: B-Raf Proto-Oncogene, Serine/Threonine Kinase; BRCA: BReast CAncer gene; cDNA: Complementary DNA (deoxyribonucleic acid); CEP: Centromere enumeration probe; cfDNA: Circulating free DNA; CISH: Chromogenic in situ hybridization; CNV: Copy number variation; CONITEC: Comissão Nacional de Incorporação de Tecnologia / National Commission of Technology Incorporation; CPS: Combined positive score; CRC: Colorectal cancer; CTC: Circulating tumor cell; ctDNA: Cell-free tumor DNA (deoxyribonucleic acid); CTLA: Cytotoxic T-lymphocyte-associated protein; ddPCR: Droplet digital PCR (polymerase chain reaction); dMMR: Mismatch repair deficient; DNA: Deoxyribonucleic acid; dNTP: Deoxynucleoside triphosphate; dPCR: Digital PCR (polymerase chain reaction); EBUS: Endobronchial ultrasound; EDTA: Ethylenediamine tetraacetic acid; EGFR: Epidermal growth factor receptor; EGJ: Esophagogastric junction; ER: Estrogen receptor; FDA: Federal Drug Administration; FFPE: Formalin-fixed paraffin-embedded tissue; FGFR3: Fibroblast growth factor receptor 3; FISH: Fluorescence in situ hybridization; FNA: Fine needle aspiration; HC: Hybrid capture; HER2: Human epidermal growth factor receptor 2; HGVS: Human Genome variation society; HS: Hotspot; HTA: Health technology assessment; IC: Immune cells; IgG: Immunoglobulin G; IHC: Immunohistochemistry; IMT: Inflammatory myofibroblastic tumor; ISH: In-Situ hybridization; IV: Intravenous; KI: Kinase inhibitor; KIT: Tyrosine-protein kinase KIT; KRAS: Kirsten rat sarcoma; LDT: Laboratory-developed test; LIMS: Laboratory information management system; LOD: Limits of detection; LSI: Locus specific identifier; MAMS: Multi-arm, multi-stage; MMR: Mismatch repair system; MSI: Microsatellite instability; MSI-H: Microsatellite instability-high; NBF: Neutral buffered formalin; NGS: Next-generation sequencing; NIH: National Institutes of Health; NRAS: Neuroblastoma RAS viral oncogene homolog; NSCLC: Non-small-cell lung cancer; NTRK: Neurotrophic tyrosine kinase; PCR: Polymerase chain reaction; PD-L1: Programmed death-ligand 1; PD1: Programmed death-1; PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; PR: Progesteronee receptor; PT: Proficiency testing; qPCR: Quantitative PCR; RAS: Rat sarcoma (a family of oncogenes); RET: Rearranged during transfection; RNA: Ribonucleic acid; ROS1: Receptor tyrosine kinase; ROSE: Rapid on-site evaluation; RT-PCR: Reverse transcription PCR; rtPCR: Real time PCR; SCC: Squamous cell carcinoma; SISH: Silver-enhanced in situ hybridization; SNV: Single nucleotide variants; SOP: Standard operating procedure; SUS: Sistema Único de Saúde / United Health System; SV: Structural variants; TAT: Turnaround time; TC: Tumor cells; TKI: Tyrosine kinase inhibitor; TMB: Tumor mutational burden; TMB-H: High tumor mutational burden; TNBC: Triple negative breast cancer; TPS: Tumor proportion score; TRK: Tropomyosin receptor kinase; TRKA: Tropomyosin receptor kinase A; TRKB: Tropomyosin receptor kinase B; TRKC: Tropomyosin receptor kinase C; US: Ultrasound; VUS: Variants of uncertain significance; WES: Whole exome sequencing; WG: Whole genome sequencing

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