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ORIGINAL PAPER

Obstacles to precision oncology: confronting current factors affecting the successful introduction of biomarkers to the clinic

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Abstract
Background Tailoring treatment strategies to individual patients requires the availability of reliable biomarkers. Despite important investment in biomarker research, few examples of successful biomarker-drug co-development are currently seen in clinical practice. The validity of a biomarker measurement may be affected by different pre-analytical, analytical and post-analytical factors. The lack of control or oversight of any of these factors may ultimately lead to failure in translating a promising research finding into clinical practice. In the present review, we put into perspective some of the obstacles to "precision" oncology, focusing on the technical and biological hurdles that may affect the validity of a biomarker result and, ultimately, the likelihood of a new targeted agent to reach the clinic.
Conclusion Biomarker application in precision oncology must consider the evolution of neoplastic disease, evaluate strengths and limitations of the platform used for the determination, and efficiently address specimen type and handling issues. In-depth analytical validation of a new biomarker test that includes evaluation of target stability should be performed before the test is used in clinical samples. More efficient sampling and use of high-sensitivity methodologies may overcome the influence of tumor heterogeneity on biomarker measurement. Clinical trials with biomarker endpoints may only be successful when multidisciplinary academic study teams are involved and results meet the highest quality standards.

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1 Introduction

The term "precision oncology" refers to the practice of tailoring treatment strategies to individual patients and/or tumors [1]. It is based on the ability to differentiate between individuals who will benefit from the application of a given therapeutic regimen and those who will not. This approach focuses on the tumor itself and its particularities. While classical histopathology remains valid for tumor diagnosis, the molecular classification of disease is becoming a prerequisite for patients to potentially benefit from novel and approved or experimental targeted therapies. An essential consideration in establishing susceptible populations is the availability of reliable and clinically approved biomarkers. The Biomarkers Definitions Working Group and American Food and Drug Administration (FDA) defined 'biomarker' as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" [2, 3]. Therefore, tissue biomarkers are expected to provide clues to better guide the diagnosis, prognosis, selection of optimal therapy, and monitoring of the disease. In drug development biomarkers are undoubtedly playing a critical role as tools to monitor drug toxicity, prove a compound mechanism of action, and predict efficacy and toxicity. Co-development of in vitro diagnostics (IVD) consequently appears key in delivering on the promise of precision oncology.

Ensuring the validity of biomarker measurements is critical for their accurate application and interpretation. Despite heavy investment in biomarker research and dissemination of published guidelines for the development, validation, and

reporting of biomarkers [4–9], few examples of molecular subtypes of cancer for which there are therapeutic approaches with proven efficacy exist [10–13]. Technical and biological factors may lead to misinterpretation, false conclusions, and impact on the likelihood of obtaining a "faithful" biomarker result [14, 15]. Certain methodological aspects are intrinsic of the assay itself, such as the robustness and reproducibility of the technology employed. Some relate to the quality of the sample used for analysis, while others depend on the complexity of tumor biology.

In this review we will tackle some of the technical and biological hurdles that may affect the accuracy of a biomarker result and, ultimately, its successful implementation into the clinic. In so doing, we will also signpost next directions towards advancing precision medicine in cancer.

2 Technical challenges

2.1 Analytical platforms

By matching targeted therapies to patients based on specific molecular aberrations, the efficacy of a drug can be evaluated in tumors expected to be "addicted" to a specific pathway.

Breast cancers with overexpression or amplification of *HER2* are sensitive to trastuzumab, a monoclonal antibody specifically targeting HER2 that inhibits the downstream signaling pathway causing tumor growth arrest [16]. Melanoma harboring the p.Val600Glu mutation in the *BRAF* gene responds dramatically in the first instance to treatment with vemurafinib [17], whilst *EGFR* gene mutant non-small cell lung carcinomas respond to treatment with gefitinib or erlotinib [18]. Conversely, most exon 12 and 13 mutations in the *KRAS* gene are predictive of a lack of response to anti-EGFR treatments, such as cetuximab and panitumumab [19].

A wide range of technology platforms may be used for tumor molecular profiling and the selection of the best approach is usually a "fit for purpose" decision balancing the desired sensitivity, the type of information requested (at DNA, RNA, Protein levels), the detection of *de novo* versus known-only alterations, and the material used for analyses, into the equation. As an example, conventional Sanger DNA sequencing, capable of both detecting the majority of mutations within a targeted region and obtaining long contiguous DNA sequence reads (>500 nucleotides), clearly suffers from sensitivity issues with mutations present at less than 15–20 % which are likely to remain undetected. In addition, sample quality (low tumor cellularity, DNA fragmentation) may significantly affect sequencing results. Despite these considerations, it continues to represent the "gold standard" for mutation detection in clinical specimens, and importantly, what is viewed to be its limiting factor (sensitivity), could very well be the reason for selecting this technology (e.g. to avoid patients with low mutation burden in a clinical study). Classical molecular pathology genotyping methods (pyrosequencing, Snapshot, LNA, etc.) offer superior sensitivity (of 99 % or even higher) and do not require long DNA fragments thus being less affected by pre-analytical sample conditions. However, the intrinsic inability of genotyping methods to cover all clinically relevant mutations contributes to any analytical insensitivity. Given this current climate, the suite of emerging next generation sequencing (NGS) technologies appear as ideally positioned to address challenges of sensitivity and coverage allowing large scale, automated, and less expensive whole genome analyses [20].

Similar to DNA analysis, gene expression-based tests such as MammaPrint® and Oncotype DX® have revolutionized the way we classify tumors, predict prognosis and make treatment decisions. Again, the decision of using genome-wide approaches such as microarrays and RNA sequencing or targeted techniques (qPCR, Transcript Analysis and Affinity Capture, Molecular bar-coding, quantitative nuclease protection assays, etc.) is highly dependent on the intended application, strengths and caveats of each particular method.

Last but not least, immunohistochemistry (IHC) is entering its fifth decade of use on formalin-fixed paraffin-embedded (FFPE) tissues and it is now routinely used in pathology laboratories worldwide. IHC is historically considered to be a qualitative method employed to observe the presence or absence of a particular protein in a tissue. Although more quantitative protein measurement methodologies exist and despite all the known difficulties in standardizing an IHC protocol, today IHC is always used more as a quantitative (or at least semiquantitative) assay to measure the level of expression of a protein [21].

For NGS to replace existing Sanger or PCR-based assays for genetic testing as well as new gene and protein expression approaches, solid data to evidence their applicability and robustness on diagnostic specimens are required [22, 23].

2.2 Pre-Analytical factors

Sequencing DNA from diagnostic tumor tissue as well as determining protein expression by IHC or gene expression by RNA microarrays, can pose specific technical challenges that may affect analytical results. The overall success and accuracy of a given approach is not only dependent on the method itself. It is also influenced by pre-analytical factors including all the variables affecting the samples from as early on as the time of the decision of procurement method up until the actual execution of the technique. Albeit a short time frame, this period harbors infinite variables, both known and unknown-both controllable and non-controllable. Molecular alterations are underway even before the sample leaves the body. The chemicals in anesthetic drugs can drive metabolic

modifications and anoxic changes derived from clamping in surgical procedures can also be observed [24]. At least sixty–one potential sources of pre-analytical variation during specimen fixation and processing have been identified and yet only 44 % of them have been documented through published results. The remaining 56 % have to-date been overlooked or unreported. Among the most studied variables are the time to sample stabilization and the duration of stabilization.

The time to stabilization or ischemia time commences from the moment that tissue is excised from the patient to the time that the specimen is placed in a stabilized state e.g. immersed in fixative or snap-frozen in liquid nitrogen. "Warm ischemia" refers to the time when blood supply stops but the specimen remains under normothermic conditions, while "cold ischemia" is the time when the sample has already been removed until fixation. Both terms are commonly referred to as "ischemia time". This period may vary from a few minutes to hours depending on local procedures, type of surgery, time to pathologist examination, etc. How ischemia time affects total proteins, phospho-proteins and nucleic acids stability has been widely discussed [25–34]. An increase in Cytokines has been demonstrated in skin wounds in as little as 10 min with additional increase over longer ischemia periods. These reactive changes may generate false increases and decreases in proteins and nucleic acids analytes [30]. For essential breast biomarkers, there are many evidences demonstrating how delay in fixation affects protein expression. Estrogen Receptor (ER) and/or Progesterone Receptor (PgR) levels have been shown to decrease with increased fixation time delay [25, 35–37]. HER2 status determination by in-situ hybridization (ISH) might also be compromised through ischemia time, with signal decrease starting at 1 to 3 h of delayed fixation [36, 38]. In the case of Ki67, ischemia time affects its expression after 4 h of excision [37, 39]; moreover, a loss of 30 to 50 % of mitotic figures has been reported when this time extends beyond 2 to 6 h [24]. Phosphorylation status of proteins can move upward or downward depending on how the pathway is affected by ischemia [27, 29, 40]. In a study comparing the expression of various markers including phosphorylated targets in pairs of core biopsies, which have minimal time to fixation, and tumor resections that undergo regular processing, a clear decrease in phospho-epitopes compared to their total protein counterparts in the surgical resections was observed [25, 26].

Just as proteins can be significantly affected by the period between excision and fixation, ischemia time may provoke changes in RNA and derived gene expression signatures [27, 31, 32, 41]. mRNA starts to degrade within the first hour of surgical removal in a time dependent manner and earlier in organs with high levels of RNAases [32]. Comparison between samples immediately preserved in RNAlater® and samples left to stand for just 30 min showed clear differences in the molecular weight of RNA fragments [32]. RIN (RNA integrity number) is not necessarily an indicator of a good sample quality and even samples with RIN >5 may be affected by delays in freezing and hypoxia [27]. Between 1 % to 20 % of all detectable genes can be modulated simply as function of collection procedures and these genes may have biological relevance [27, 42]. Interestingly, one article claimed that non-fixed tissues can be kept on ice for hours without perjury of RNA quality and gene expression [33].

The type and duration of stabilization induce modifications in the tissue that has to be considered when selecting the technology platform for biomarker analyses. Although snap freezing of a tissue sample is unanimously considered the optimal stabilization mechanism through which to preserve nucleic acids and proteins, many obstacles exist impeding its use as a standard stabilizer outside of the research context. An alternative to cryopreservation is the use of chemical fixation. There are two major types of chemical fixatives; precipitation and cross-linking. The first includes ethanol, methanol and acetone, and retains good molecular and cellular substances but lacks conservation of morphological detail. Cross-linking fixatives such as formaldehyde and glutaraldehyde however, conserve morphology while they compromise molecular preservation. Formalin fixation and paraffin embedding currently represents the most widely used stabilization mechanism for tissues in routine diagnostic pathology and biomarker developers must confront the magnitude of alterations resulting from this fixative. In the case of IHC, it has been shown that type, fixative buffer presence or absence and percentage, volume, pH, temperature and time of formaldehyde fixation affect the expression of biomarkers at different levels [43, 44]. Formaldehyde fixation induces the formation of cross-links between proteins and between proteins and nucleic acids, which may result in the masking of epitopes. Both under and over-fixation may lead to a false result in protein expression with IHC, influencing the likelihood of false negative or positive results [43, 45]. Reduced fixation time in formaldehyde solutions followed by the transfer to alcohol results in a mixture of cross-linking fixation and a coagulation fixation induced by alcohol tissue penetration. Antigen retrieval (usually required for epitope demasking in IHC), on an underfixed tissue results in high background, section fall-off, holes and folding in the tissue, altered nuclear and chromatin image, among other alterations [43]. Overfixation, on the other hand, may create excessive cross-linking that will mask epitopes that will not be exposed using regular antigen retrieval protocols [46]. A comprehensive review on pre-analytical variables affecting immunohistochemical protein detections has been recently published [28].

Nucleic acids extracted from frozen material yield higher quality and quantity than those extracted from FFPE samples, which are usually fragmented and cross-linked [47]. RNA studies based on FFPE material have triggered major debate regarding the suitability of FFPE tissue samples for gene

expression analyses. Fixation in formalin significantly affects the quantity and quality of extracted nucleic acids compared to snap freezing and the level of damage is even higher when dealing with non-buffered formalin compared to neutral buffered formalin [31, 32, 48]. The size of DNA extracted from FFPE decreases with increased fixation time. Heavily fragmented DNA as a consequence of overfixation and chemical modifications induced by fixation might ultimately result in either artifact mutations or false negative mutations with a highly detrimental effect on treatment decision-making based on genetic criteria [49].

2.3 Sample heterogeneity

Sample heterogeneity is determined by the degree of "contamination" of a tumor sample by healthy bystander cells (e.g. normal epithelial cells, normal stromal cells, inflammatory cells). The degree of normal contamination may obscure the real frequency of a mutant allele (by genotyping) or the gene expression profiling of the tumor (by microarrays). As an example, if normal and tumor cells are present in similar proportions in a sample, the existence of a mutated gene in one allele of tumor cell will be reduced at 25 %. Some techniques are more sensitive to tumor contamination than others. For instance, the mutant allelic fraction has to be ≥20 % in mass spectrometric genotyping to ensure a reliable reading, while deep sequencing can retain sensibility when the mutated allele is present in ≤5 % of the tested material [49]. Sample heterogeneity is not an issue when using high-resolution techniques such as IHC that allow precise identification of the cellular component in a tumor expressing the target biomarker. To overcome this limitation when high purity is needed, microdissection strategies have previously been successful. Many techniques have been described including selective ultraviolet radiation fractionation, selective laser ablation, and manual microdissection [50], all with the goal of purifying or, at least, enriching the sample for the component of interest. However, the required level of purity is highly dependent on the biomarker/s under study and the technology used, thus making it impossible to define a unique cellularity cut-off. 70 % purity was considered enough to yield sufficient mRNA quality for gene expression profiles in gastric cancer [51]. In a recent article reporting a procedure for simultaneous isolation of high quality DNA, RNA, miRNA and proteins, a similar cut off of 70 % of tumor purity was recommended for solid tumor, and also to avoid samples with >20 % of necrosis [52]. For mutation analyses using a highly sensitive method, 10 % tumor cellularity content may be sufficient for mutation detection.

In addition to mechanical approaches, some groups have also established the use of specific genes to differentiate between tumor and stromal compartments. Shukla et al. reported the use of Cytokeratin 8 and PSA, expressed only in the epithelial compartment, to compare with HGF and TIMP3 only present in the stroma, in prostate cancer [53]. Otsuka et al. described a ratio between a gene only expressed in gastric tumor cells, *cytokeratin 19*, and a housekeeping gene such as *GAPDH* present in all the cells, as a way to determine purity [51]. Recently, a gene expression signature called ESTIMATE (Estimation of Stromal and Immune cells in Malignant Tumors using Expression data) has been proposed as capable of inferring tumor purity based on stromal and immune component levels [54].

2.4 Sample type: core biopsy versus surgical samples

Accuracy of a biomarker measurement may be affected by the sample type used (e.g., core-cuts, punch biopsies, excisions). Significant differences in biomarker expression have been observed between core biopsies and excision samples [40, 55]. Comparative analysis of Ki67, ER, PgR, HER2, p-Akt and p-Erk1/2 in core-cuts taken from surgical breast cancer specimens immediately after resection and routinely fixed resection specimens, showed extreme loss of phosphomarkers in routine fixation of resection specimens [40]. Suboptimal fixation is likely to occur in large samples due to slower fixative penetration when compared with smaller ones, the latter usually resulting in an overall superior sample quality. Sample size differences inherent to the sampling method are accountable for possible discrepant results when dealing with highly morphologically and molecularly heterogeneous tumors. The amount of evaluable tumor decreases as sample size reduces. Results consequently obtained from small biopsies may not be sufficiently representative of the entire complexity of tumors. The decision regarding the type of sample to use, again, supposes a balance of pros and cons when considering each methodology.

3 Biological challenges

3.1 Tumor heterogeneity

Phenotypic and genetic variations exist not only between tumors (intertumoral heterogeneity) but also within individual tumors (intratumoral heterogeneity). Different cell populations may coexist within the same sample when evaluated histopathologically by optical microscopy. These differences extend beyond morphology and homogeneous tumors can apparently show an extensive genetic diversity in the form of intermingled or spatially separated subclones. Clonal evolution along with genomic instability provides a framework for tumor heterogeneity [56, 57]. This process can be either linear or branched. In the case of the former, a favored subclone/s harboring growth advantages expand, resulting in

low heterogeneity. In the branched evolution model, distinct subclones evolve in parallel, resulting in extensive subclonal diversity [58]. In the case of breast cancer, intratumoral heterogeneity has been clearly documented for ER, PgR, and HER2, both by IHC and FISH [59–62]. In particular, block to block heterogeneity for ER expression ranges up to 81 %, which has also been apparent within individual slides [60]. Using an automatic analyzer, the estimated number of 20× fields to overcome heterogeneity varied depending on breast cancer biomarkers, ranging from 3 to 14 for the most heterogeneous one [61]. In lung cancer, a high variability in the percentage of positively *ALK* rearranged cells within the tumor has been described [63]. Similarly, activating *EGFR* mutations were found to display intratumor heterogeneity and spatial discordance in 6.3–28 % of cases [64]. Conversely, *PIK3CA* mutations are rarely heterogeneously distributed [65].

Biomarker expression or distribution within a tumor is not static but may change throughout the course of disease as a consequence of clonal evolution of the original founding population or change in tumor subclone architecture [66, 67]. In situ carcinomas are not necessarily a clear reflection of their corresponding invasive tumors neither morphologically, nor in terms of biomarker expression. The HER2 oncoprotein as well as its gene amplification have been described in ductal in situ carcinoma of the breast in higher frequency than in its invasive counterpart [68, 69]. *KRAS* gene mutations have also been observed in higher percentages in pre-malignant as well as confined lesions than in the invasive components or at tumor progression. This phenomenon was described both in colorectal and lung cancers and might indicate that either gene alteration is somehow lost during progression, or that these early lesions do not follow a linear progression [70, 71]. On the contrary, *PIK3CA* gene mutations seem to appear early in tumorigenesis and are present essentially in the same percentages in in-situ lesions as well as invasive breast cancer tumors [65, 72]. Primary tumors and metastatic counterparts do often present with different features, both genotipically and phenotypically. The expression of many biomarkers has been reported to change when comparing primary and metastatic pairs. Discordance rates between primary breast cancer and its metastases in ER, PgR, and HER2 were 16, 40 and, 10 %, respectively in one series [73]. In another series, HER2 expression by IHC changed from positive to negative in the metastasis in 24 % of 182 paired samples analyzed; more importantly, the administration of trastuzumab was not associated with HER2 loss in metastasis [74], confirming a previous publication where negative to positive conversion observed in 15 % of cases had no relationship to anti-HER2 therapy [75]. Two other series revealed differences in expression of HER2 from primary to metastatic cancer. Lower et al. reported a change of up to 33 % from primary to metastasis, 23.5 % from positive to negative [76], while Fabi et al. showed a change from positive to negative HER2 in the metastatic site in almost 9 % of cases in a series of 137 breast cancer patients [77]. Based on these evidences, recommendations for retesting breast cancer metastases have been issued [78], particularly in patients with HER2 positive primary breast tumors. In the case of *PIK3CA* and *PTEN*, there are conflicting reports with regards to concordance between paired primary and metastatic breast cancer. Results from two groups showed a change in *PIK3CA* mutational status comparing primary tumors to asynchronous metastases in up to one third of patients, favoring change from wild type (WT) to mutated at progression [79, 80]. In contrast, Kalinsky et al. found >90 % concordance in *PIK3CA* status between primary tumor and either lymph node or distant metastasis. In addition, they did not find any evidence of heterogeneity in PIK3CA status in primary tumors and suggested that the apparent lack of concordance with other reports might be due to technical issues [65]. Daneshmand et al. reported that the *PIK3CA* mutation status in bone metastases samples appears to reflect *PIK3CA* status in the primary tumor [81]. For PTEN, a lower frequency of protein loss has been observed in metastases compared to primary breast tumors, with a 26 % discordance reported by Gonzalez-Angulo et al. [79, 80].

In colorectal cancer, the mutational status of *KRAS*, *BRAF*, and *PIK3CA* in matched primary tumors and distant metastases was highly concordant (≥90 %) in two independent series [82, 83]. Conversely, heterogeneity between primary tumors and lymph node metastases was found in 31 % (*KRAS*) and 13 % (*PIK3CA*) of the cases. Discordant results between primary tumors and metastases could markedly be reduced by testing the additional tumor samples [82, 83].

The existence of this inter- and intra-tumoral heterogeneity imposes some type of interaction between clones, and the behavior of the parts somehow influence tumor development, initial response and subsequent resistance to specific treatments. The co-existence of multiple aberrations within the same tumor and the architectural re-shaping that the tumor undergoes during its evolution, have significant implications in the choice of biomarkers to guide clinical decision-making in cancer medicine. *BCR-ABL* translocation in chronic myelogenous leukemia renders it exquisitely sensitive to imatinib [84]. The same was described of the presence of *EGFR* alterations and response to tyrosine kinase inhibitors [85]. However, a proportion of patients that relapse show the presence of "new" genetic mutations that were ignored or not originally detected [86–89]. Clonal selection is considered to be one of the reasons behind therapy resistance in initially responding cases; these resistant clones might be acquired or already be present in the original tumor and progress as the reigning clone post therapy. In such a scenario, longitudinal tumor sampling over the disease course and throughout treatment as well as the use of high sensitive techniques capable of

detecting low frequency events might be compelling to predict therapy outcome.

4 Biomarkers in clinical trials

4.1 Sample accrual

Clinical trials of investigational targeted agents represent the ideal setting for the translation of a research biomarker to a companion diagnostic. Both the availability of high quality biospecimens and carefully validated assays are crucial for the successful biomarker-agent co-development. Suboptimal tissue handling and/or accrual may lead to inconclusive correlative studies, especially when the expected frequency of the biomarker alteration is low in the target population. Specimen accrual is highly variable and depends on the study design, type and amount of material required, number of centers involved, as well as sample collection protocols, among other considerations. Attrition factors include low rate of success in collection, heterogeneous processing, insufficient material and quality. Demonstration of the link between *EGFR* mutations and sensitivity to gefitinib and erlotinib in lung cancer has been hampered by low tissue accrual (<24 %) in the major trials involving EGFR tyrosine kinase inhibitors (IDEAL-1, IDEAL-2, INTACT-1, INTACT-2, TRIBUTE, TALENT, BR.21, ISEL and INTEREST). The retrospective nature of these studies and the absence of specific tissue requirements have been indicated as possible reasons for low sample availability. On the contrary, tissue accrual in colon cancer trials has proven suitably efficient (45–92 % patients had suitable tissue available for analyses) to validate the presence of *KRAS* mutation as a negative biomarker of response to anti-EGFR therapies [90]. In the NeoALTTO trial, a large neoadjuvant Phase III multicenter, multinational clinical trial collecting serial samples (plasma, serum, frozen and FFPE specimens) from patients with breast cancer from 86 sites in 23 countries, the overall missing sample rate was extremely low (9.5 %) and the evaluable sample population was 71 and 80 % for downstream RNA- and DNA-based analyses, respectively. These results were achieved thanks to careful upfront logistical and technical planning (author's unpublished observations). Similarly, in the TBCRC006 trial, accomplishment in specimen accrual was 88 %, which varied from 95 % at baseline, to 78 % at week 8 [91].

4.2 Local versus central laboratory testing

Discordant rates between laboratories are unacceptably high in many studies which pose the question regarding the validation and reproducibility of the assays, and the need to establish agreed methods and criteria prior to commencing the trial. Triple negative status in breast cancer patients enrolled in the CIBOMA/2004-01/GEICAM/2003-11 study was discordant between local and central laboratories in 9 % of the cases. Most of the discrepancies involved ER and PgR status (71 %), while HER2 differences represented 22 % of cases (Ruiz-Borrego, unpublished data). In the Breast Intergroup Trial N9831, 26 % of the first 119 patients initially enrolled based on local HER2 positive results, failed to be confirmed centrally [92] which led to a change in the eligibility criteria. A posterior publication surrounding the same trial, reported the agreement rates between local and central laboratories: 88.1 % for FISH, and 81.6 % for IHC, using the same methodologies [93]. In ALTTO trial, two different academic laboratories performed the central confirmation of HER2, ER and PgR status [94]. Disagreement rates varied from 5.8 to 14.5 % for HER2; and 3.4, and 21.4 % for ER, depending on the laboratory. The subsequent ring study performed by the 2 central laboratories to identify reasons of discrepancy, showed how the use of even slightly different assay methods may yield different results, even between experienced laboratories. High inter-laboratory and inter-observer Ki67 staining and interpretation variability has been extensively proven with two large series showing serious discrepancies in staining and evaluation. So much so, that the need for standardizing this biomarker evaluation is not a point for discussion [39, 95, 96]. In a clinical trial setting, centralized testing performed in accredited academic or commercial laboratories represents the ideal solution to guarantee homogeneity and quality of biomarker results. Outside clinical studies however, the lack of experience with the new test and absence of external quality assurance programs may represent a challenge for its rapid implementation in routine practice.

5 Perspectives and Conclusions

The success of targeted therapies will depend on addressing the many limitations that previous experiences in the field of biomarker discovery and stratified medicine approaches have emphasized. We should consequently anticipate new future directions aimed at addressing and resolving these issues.

First, what has been considered "gold standard" up to now may soon become obsolete due to emerging more accurate, quantitative, and less expensive technologies rapidly moving from the research setting to the bedside. Targeted next-generation sequencing assays such us Foundation One™ or clinical proteomics (Veratag™, OncoplexDx™, among others) are now available as clinical products at the service of personalized oncology. Multiplex diagnostics from the same small amount of FFPE tissue represent a new option for pathologists, clinicians and patients. They provide fully informative genomic and/or proteomic profiles of all known

actionable genes/proteins alterations in a tumor, expand treatment options for patients, and optimize the use of specimens.

Second, the pathology community needs to react promptly to this new paradigm since it assumes a crucial role in understanding and controlling pre-analytical and analytical variables that may affect test results. Obsolete routine laboratory procedures designed to provide a histological definition of the disease may have to adapt to the tissue quality stardards required for the molecular classification of tumors - now an essential pre-requisite for patients to have access to new "smart drugs". Similarly, subjective, qualitative or semi-quantitative analyses on tissue specimens using IHC will probably be replaced by quantitative biomarker measurements that appear to better predict treatment efficacy [97, 98].

Third, the accurate determination of positivity or negativity of a particular biomarker may not be enough when dealing with biological challenges such as tumor heterogeneity. Efforts aimed at better understanding the science behind the origin and clonal evolution of cancer will help delineate more efficient tumor sampling methods and the required analytical sensitivity for selecting the best technology platform for biomarker analyses. Repeated biopsies taken on treatment and at progression will form an integral part of personalized oncology by providing real time monitoring of the disease. This should facilitate a more effective intervention by changing the therapeutic strategy even before signs of clinical and/or radiological resistance appear. To achieve this goal, tumor biopsy collection procedures should be improved to increase the rate of adequate samples for translational analyses. The likelihood of success of these studies is strictly linked to the level of commitment and involvement of key figures such as surgeons, interventional radiologists, oncologists, and pathologists, among others. The Breast International Group (BIG)-sponsored Groups and the National Cancer Institute (NCI)-sponsored North American Cooperative Groups have developed recommendations aimed at promoting identifiable standards for specimen collection and handling within and across breast cancer trials. These include suggestions for collection timepoints, number of biopsies, and processing of material in order to maximize results from diagnostic testing and research [99, 100]. Similar efforts should be made for other cancer types which may present specific issues.

To conclude, we have reviewed the challenges that are integral to the implementation of a new biomarker into the clinic. Biomarkers in precision oncology must take into account the spatial and temporal evolution of neoplastic disease, overcome the potential bias in tissue selection and sampling, as well as the analytical methodology used when interpreting potentially conflictive results. The benefit will be the development of robust molecular assays that can improve selection of patients for targeted therapies.

Conflict of Interest Authors do not have any conflict of interest.

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