Pragmatic issues in biomarker evaluation for targeted therapies in cancer

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Abstract | Predictive biomarkers are becoming increasingly important tools in drug development and clinical research. The importance of using both guidelines for specimen acquisition and analytical methods for biomarker measurements that are standardized has become recognized widely as an important issue, which must be addressed in order to provide high-quality, validated assays. Herein, we review the major challenges in biomarker validation processes, including pre-analytical (sample-related), analytical, and post-analytical (data-related) aspects of assay development. Recommendations for improving biomarker assay development and method validation are proposed to facilitate the use of predictive biomarkers in clinical trials and the practice of oncology.

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Introduction

The development of cancer therapies is increasingly dependent on our understanding of tumour biology, and biomarkers—especially predictive biomarkers—are crucial tools in the field of personalized medicine and health economics, in particular, as they enable definition of the populations of patients who are most likely to benefit from targeted therapies. More-effective patient selection than is possible at present is mandatory to improve the success rate of new therapies, which are sometimes prohibitively expensive, and thereby increase their cost–utility; thus, delineating reliable predictive biomarkers is essential if we are to achieve this objective.

One commonly used definition of a biomarker is a measurable indicator that is used to distinguish precisely, reproducibly and objectively either a normal biological state from a pathological state, or the response to a specific therapeutic intervention.[1] In fact, biomarkers are used for numerous purposes: to predict survival (prognostic biomarkers); to assess drug safety and evaluate target engagement and the immediate consequence on biological processes (pharmacodynamics biomarkers), to identify patients who are more likely to benefit from a treatment (predictive biomarkers; more generally termed companion biomarkers when associated with a specific therapeutic agent); to predict outcome given the response to therapy (surrogate biomarkers); and to monitor disease progression or therapeutic efficacy (monitoring biomarkers). Identification and widespread use of biomarkers will help ensure that patients receive the best possible therapeutic strategies, thereby avoiding unnecessary treatments and associated toxicities, and eventually reducing total health costs.

Competing interests
The authors declare no competing interests.

Most cancer therapies, especially those developed in unselected patient populations, offer only limited clinical benefits. As an example, in phase III trials that enrolled patients with metastatic colorectal cancer (mCRC), bevacizumab was associated with a median overall survival advantage of 1.4–4.7 months when added to first-line chemotherapy and 2.1 months with second-line chemotherapy, for a median overall survival duration of more than 20 months; therefore, the cost–utility benefit of adding bevacizumab to chemotherapy is potentially marginal, in some cases.[2–4] Thus, biomarkers that clearly define a subgroup of patients with mCRC who are most likely to benefit from the addition of bevacizumab to chemotherapy would enable the use of this agent to be focused more effectively, which would be equally important for patients and health payers. Considering that biomarkers are nowadays integrated into most drug development programmes, from target identification and validation to clinical practice, robust measurements and assay validation for analyses of biological samples have become essential. Without a robust methodological foundation and pertinent biological interpretation, the number of reliable biomarkers that emerge will probably be limited, and their potential utility in the evaluation of novel treatments and customization of clinical strategies will be underexploited. In fact, despite the large volume of research that has been devoted to identifying cancer biomarkers and the vast quantity of candidate biomarkers studied, only a small number of cancer biomarkers per year have been approved for use by the FDA in the USA and the European Medicine Agency (EMA).[5]

Development and validation of biomarkers is as difficult as the development and approval of a new drug; indeed, approximately 30–50% of biomarkers are

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Key points

- Predictive biomarkers are essential tools with regard to personalized medicine and health economics, and are crucial to improve the success rate of new therapies
- Implementation of biomarkers into clinical practice presents biological, clinical and logistical challenges, in particular, relating to standardization across multiple countries and clinical practices
- During biomarker development, robust laboratory methodology is necessary at all analytical phases, from pre-analytical (sample definition, handling and processing) to analytical (data and quality-control recording) and post-analytical (data reporting and interpretation)
- A series of recommendations can be made to increase biomarker reliability and facilitate development of predictive biomarkers that can ultimately be used to provide benefit for patients with cancer

Box 1 | Fit-for-purpose biomarker method validation: an overview

Adopting a fit-for-purpose approach to method validation for biomarker assays relies on acknowledgement of the fact that evaluation of the technical performance of an assay should reflect the intended purpose of the biomarker and the nature of the bioanalytical methods used to generate data. In the development process of biomarkers, the intended purpose for method validation will be intertwined with the development phases of a potential drug. As pharmacodynamic, monitoring, prognostic, predictive or surrogate biomarkers have different intended use, it follows that the stringency of the assay will depend on the intended use of the biomarker, and will increase with each developmental phase, from discovery to validation for the intended purpose. Stringency in biomarker assay validation also needs to integrate the nature of the bioanalytical methods—whether quantitative or qualitative. As proposed by Lee and colleagues,[12,185] bioanalytical methods can be divided in four categories: definitive quantitative assays, such as the use of mass spectrometry assays to quantify circulating insulin concentrations; relative quantitative assays, such as enzyme-linked immunosorbent assays (ELISAs); quasi-quantitative assays, such as real-time quantitative reverse-transcription PCR (qRT-PCR)-based assays; and qualitative assays, which include most immunohistochemistry assays.

According to the position paper by Lee and colleagues,[12] the fit-for-purpose biomarker assay validation can be separated in four continuous iterative activities:

- The pre-validation process that defines the intended purpose of the biomarker, considering pre-analytical variables and bioanalytical method feasibility
- The exploratory validation process that assesses the basic assay performance
- The advanced validation process that characterizes the formal performance of the assay with regard to its intended use
- The in-study validation process that ensures that the assay method performs robustly across studies according to predefined specifications and facilitates the establishment of definitive acceptance criteria

coupled to drug development programmes and only 3–5% reach the clinic.[6–9] When co-developing a drug and a biomarker, it is relevant to use a 'fit-for-purpose' approach to biomarker method validation (Box 1), in which methods can be refined throughout the development phases of the experimental agent.[10–12] This key conceptual methodology enables the developers to focus on the specific requirements of biomarker method validation in a timely manner, depending on the purpose of the biomarker (predictive versus pharmacodynamic, for instance), the type of bioanalytical method and purpose of the clinical trial, and the information that needs to be collected as part of the drug-development process. In light of the high drop-out rate in biomarker development, a fit-for-purpose strategy for method validation might also be economically relevant to progress biomarker assays and achieve regulatory approval. The importance of robust methodology is heightened by the fact that new drugs often display modest benefits and that many potential biomarkers—such as gene-copy number and gene and/or protein expression—are continuous variables, the application of which relies heavily on interpretation of data, with the risk of subjectivity, to establish thresholds.[13] Hence, robust and validated biomarker cut-points that can accurately quantify drug benefits, stratify patient populations, and predict patient responses to treatment are required. A key goal is, therefore, to classify cancers not only according to their molecular profiles (such as mutational status), but also, more importantly, based on their response to therapies (that is, according to individual biomarkers or composite clinical, radiological, and/or biological biomarkers that clearly define the beneficial therapeutic windows of a treatment). A combination of cancer molecular and pharmacological profiles is likely to be the most-successful strategy for guiding therapeutic interventions.

Recognizing the above challenges and the absence of robust standards for evaluation and adoption of biomarkers, an ongoing trend—involving academia, professional organizations, and industry—has been to improve standardization of procedures for biomarker development in oncology.[14–16] Although these joint efforts have yielded some technological improvements in terms of specimen acquisition and processing, assay automation, production of qualified reagents, and standardization of laboratory procedures, much work remains to be done to achieve universal and robust methodologies. In particular, although the quality and consistency of technological assays have improved, less progress has been made in ensuring the quality of biospecimens and harmonization of tissue collection, processing and storage procedures, attributable largely to the long-standing success of formalin-fixed paraffin-embedded tissue analysis as the standard in diagnostic pathology. Although continued technological advancement would be beneficial, further effort should be made to standardize methodologies as well as quality control and quality assurance procedures, and to rigorously apply such standards in clinical practice.

Herein, we discuss different technical and logistical challenges that must be addressed in the process of standardization of biomarker measurements. Recognizing these challenges, we also outline key considerations for validation of pre-analytical, analytical, and post-analytical processes in biomarker assays. Recommendations for optimizing biomarker evaluation are provided.

Challenges for standardization

Continuing progress in the widespread implementation of valid biomarkers into clinical trials and clinical practice as elements of the development of effective targeted therapies presents biological, clinical, and logistical challenges. The challenges in each of these areas are discussed in the following sections.

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Biological challenges

A major challenge in biomarker development is the inherent biological complexity underlying tumour response to treatments (treatment sensitivity, and primary and/or acquired treatment resistance). A complex network of multiple interacting molecular pathways, with adaptive feedback and cross-talk loops, clearly hinder the ability of a single biomarker to capture responses of the system as a whole. Thus, to improve upon the limited predictive power of individual biomarker candidates, a panel of multiple markers will generally be required to generate more-sensitive and more-specific composite biomarkers for characterizing system functions, and predicting treatment responses and outcomes. Further biological obstacles are the multiple diverse functions of potential drug targets, as well as the various mechanisms of action and biological effects of individual treatments (cytostatic or cytotoxic), each of which necessitate the development of evidence-based and disease-tailored biomarkers. In addition, intratumoural heterogeneity—characterized by both genetic diversity of tumour cells and the heterotypic matrix comprised of tumours cells, nontumour cells of different types, and the extracellular matrix—represents a universal feature of solid tumours that must be factored into analyses in the search for robust predictive biomarkers.

The challenge posed to biomarker standardization by intratumoural heterogeneity is emphasized by differences in their expression between primary and metastatic tumours. Several studies have performed comparative analyses of gene expression and mutation status of key biomarker oncogenes—*HER2*, *KRAS*, and *BRAF*—between primary and metastatic sites (Tables 1 and 2).[17–76] Overall, the clinical concordance between expression of these genes in primary tumours and disseminated tumour cells ranged between 53% and 100%; however, most studies demonstrate concordance rates of 85–100% (55 out 75 concordance evaluations; Tables 1 and 2). We identified a series of 35 evaluations that compared HER2 expression by immunohistochemistry, *HER2* amplification using fluorescence *in situ* hybridization (FISH), or both, between primary tumours and metastases or recurrence lesions; among these evaluations, the overall concordance estimation was around 87% (Table 1). Given that HER2 positivity occurs in roughly 25% of primary breast tumours (23.2% based on the data in Table 1), however, concordance in the population of patients with HER2-positive primary tumours might be reduced due to the predominance of HER2-negative tumours with HER2-negative disseminated disease. For example, considering the pooled data of Curigliano *et al.*[23] and Dieci *et al.*,[24] we calculated that HER2 was expressed in 25.8% of the primary tumours, and 31.6% of the combined primary and disseminated tumour samples. The overall concordance in the whole population across these two studies was 86.9%, whereas the concordance of HER2-positive metastases in patients with HER2-positive primary tumours was 72.0%, and this concordance was further decreased to 58.7% when both HER2-positive primary and disseminated tumours were considered. Furthermore, two studies in the series that studied only patients with HER2-positive primary tumours displayed a calculated overall concordance of 78.6% (based on a 76.4% concordance in one study of 182 patients,[38] and 84.9% concordance in the second study of 66 patients[47]). Overexpression of HER2 assessed by immunohistochemistry had a 100% concordance rate in only one study.[41] The difference in concordance between FISH-detected *HER2* amplification and HER2 overexpression by immunohistochemistry is further elaborated on in the 'Analytical standardization' section of this manuscript. Similarly, in a series of 40 studies comparing the mutation status of *KRAS* or *BRAF* between primary tumours and metastases, the overall concordance reached 93% (Table 2). Sequencing was the most-frequently applied method of biomarker analysis, and even though the overall degree of the agreement was high (66–100%), only four out of the 40 studies we reviewed reported 100% concordance (Table 2). Details on the techniques used for detection of *KRAS* or *BRAF* mutations are beyond the scope of the Review and have been published elsewhere;[77–80] however, the methodologies used in determining any fraction of a biomarker alteration among studies—specific technologies or cut-off points, for instance—are critical for a pertinent biomarker evaluation and, therefore, are discussed further herein.

Among recognized positive or negative predictive biomarkers, genetic alterations such as mutations, amplifications, or translocations seemed to be more concordant between primary tumours and associated metastases than protein or gene-expression levels or signatures.[61] This observation probably reflects the introduction of increased analytical variation in gene-expression methodologies and the complexity of protein biochemistry, including post-translational modifications and catabolism. However, whereas high concordance occurs for many recognized genetic biomarkers (including recurrent *TP53* mutation), such is not the case for many genetic modifications that are nonrecurrent and probably represent passenger alterations.[81] Given these findings, the relevance of primary resection specimens to evaluate biomarkers when planning treatment in the metastatic setting has been questioned. In fact, during the course of the disease, a number of factors could potentially influence biomarker concordance (Table 3), and might, therefore, affect biomarker evaluation and challenge therapeutic decisions. It has long been recognized that biomarker status can be discordant due to inherent intratumoural and intertumoural heterogeneity, clonal evolution during tumour progression due to genomic instability, or treatment effects that result in elimination of sensitive tumour cells and/or adaptation of tumour cells in response to therapeutic agents. As a result, the discrepancy between the first tumour evaluation (typically based on resected primary tumour or core needle-biopsy specimens) and assessments of subsequent samples, either from the same site or distant metastatic sites, might be evident and could have been introduced by intervening treatment.[82–84] Pre-analytical and analytical factors, such as the sensitivity of the

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Table 1 | HER2 status* concordance in matched primary tumours and metastases from patients with breast cancer

Study (year of publication)	Method of biomarker assessment	Number of patients			Timing of metastasis	Concordance rate[‡] (%)
		n_{total}	$n_{primary}$ (%)	$n_{disseminated}$ (%)		
Aktas *et al.* (2011)[17]	IHC	86	7 (8)	27[§] (32)	MC	79[‖]
Amir *et al.* (2012)[18]	FISH	83	10 (12)	14 (17)	MC	90
Aoyama *et al.* (2010)[19]	FISH	60	18 (30)	15[¶] (25)	SC	92
Botteri *et al.* (2012)[20]	IHC and FISH	60	17 (28)	25 (42)	MC	87
Cardoso *et al.* (2001)[21]	IHC	334	36 (11)	40[¶] (12)	SC	98
Chang *et al.* (2011)[22]	IHC and FISH	56	15 (27)	18 (32)	SC and MC	88
Curigliano *et al.* (2011)[23]	IHC and FISH	172	54 (31)	44 (26)	MC	86
Dieci *et al.* (2013)[24]	IHC and FISH	119	21 (18)	27 (23)	SC and MC	88
Duchnowska *et al.* (2012)[25]	IHC and FISH	119	58 (49)	61 (51)	SC and MC	86
Fabi *et al.* (2011)[26]	IHC and FISH	137	25 (18)	36 (26)	MC	90
Fehm *et al.* (2009)[27]	RT-PCR	58	9 (16)	22[§] (38)	MC	53
Fuchs *et al.* (2006)[28]	IHC and FISH	48	8 (17)	6[¶] (13)	MC	79
Gancberg *et al.* (2002)[29]	IHC	93	13 (14)	19[¶] (20)	MC	94[‖]
Gancberg *et al.* (2002)[29]	FISH	68	16 (24)	17[¶] (25)	MC	93
Gong *et al.* (2005)[30]	FISH	60	20 (33)	18[¶] (30)	SC and MC	97[‖]
Guarneri *et al.* (2008)[31]	IHC and FISH	75	14 (19)	22 (29)	MC	84
Jensen *et al.* (2012)[32]	IHC and FISH	114	10 (9)	16 (14)	SC and MC	91
Lear-Kaul *et al.* (2003)[33]	IHC and FISH	12	4 (33)	5[#] (42)	MC	92
Lindström *et al.* (2012)[34]	IHC and FISH	104	29 (28)	26[#] (25)	MC	86
Lower *et al.* (2009)[35]	IHC	382	140 (37)	87[¶] (23)	MC	66
Macfarlane *et al.* (2012)[36]	IHC and FISH	154	29 (19)	25[¶] (16)	MC	95
Montagna *et al.* (2012)[37]	IHC	174	51 (29)	52[#] (30)	MC	96
Niikura *et al.* (2012)[38]	IHC and FISH	182**	182 (100)	139 (76)	SC and MC	76
Regitnig *et al.* (2004)[39]	IHC	31	3 (10)	6[#] (19)	MC	77
Regitnig *et al.* (2004)[39]	FISH	18	2 (11)	6[#] (33)	MC	78
Santinelli *et al.* (2008)[40]	IHC and FISH	54	16 (30)	13[‡‡] (24)	SC and MC	94[‖]
Santinelli *et al.* (2008)[40]	IHC and FISH	65	12 (18)	17[#] (26)	SC and MC	78
Shimizu *et al.* (2000)[41]	IHC	21	8 (38)	8[¶] (38)	MC	100[‖]
Simon *et al.* (2001)[42]	IHC and FISH	125	31 (25)	24[¶] (19)	SC	95[‖]
Simmons *et al.* (2009)[43]	FISH	25	4 (16)	6 (24)	MC	92
Thompson *et al.* (2010)[44]	IHC and FISH	137	14 (10)	16 (12)	MC	97
Vincent-Salomon *et al.* (2002)[45]	IHC	44	11 (25)	9[¶] (20)	SC	95[‖]
Wilking *et al.* (2011)[46]	IHC and FISH	151	43 (28)	41 (27)	SC and MC	90
Xiao *et al.* (2011)[47]	IHC and FISH	66**	66 (100)	56[¶] (85)	MC	85
Zidan *et al.* (2005)[48]	IHC	58	14 (24)	20[¶] (34)	MC	86
All	NA	NA	NA	NA	NA	87[§§]

*Overexpression or amplification. [‡]As calculated by the authors of this manuscript or within the original publication, unless otherwise noted. [§]Circulating tumour cells. [‖](Over)-estimated using the calculation $1-[(n_{primary}-n_{disseminated})/n_{total}]$. [¶]Lymph-node or distant metastases. [#]Recurrence or distant metastases. **HER2-positive population only. [‡‡]Lymph-node involvement only. [§§]Calculated using the equation $[\Sigma(n_{total} \times \text{Concordance rate})]/\Sigma n_{total}$. Abbreviations: FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; MC, metachronous; NA, not applicable; $n_{disseminated}$, number of patients with HER2-positive disseminated cells; $n_{primary}$, number of patients with a HER2-positive primary tumour; n_{total}, number of patients in the study; RT-PCR, reverse-transcription PCR; SC, synchronous.

laboratory method used for biomarker evaluation, might also be involved in such discrepancies, and can have important clinical implications. For example, detection of rare *KRAS*/*NRAS*-mutant clones that will ultimately become predominant can predict eventual resistance of cancerous lesions to EGFR-targeted monoclonal antibody therapies.

Clearly, both laboratory techniques as well as biomarker heterogeneity (with regard to expression and types of mutation), must be considered when incorporating biomarkers, especially predictive biomarkers, into future clinical trials or routine patient care. Detection of selected mutations in circulating cell-free DNA (cfDNA) by BEAMing (beads, emulsion,

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Table 2 | *KRAS* and *BRAF* mutation status concordance in matched primary tumours and metastases in CRC

Study (year of publication)	Method of biomarker (mutation status) assessment	Number of patients			Timing of metastasis	Concordance rate* (%)
		n_{total}	$n_{primary}$ (%)	$n_{disseminated}$ (%)		
KRAS						
Albanese *et al.* (2004)[49]	SSCP	30	14 (47)	13[‡] (43)	SC and MC	70
Al-Mulla *et al.* (1998)[50]	ASO/Seq	47	NR	NR[‡]	NR	83
Artale *et al.* (2008)[51]	NR	48	11 (23)	12[‡] (25)	SC and MC	94
Baldus *et al.* (2010)[52]	Seq/PyroSeq	55	29 (53)	16[§] (29)	NR	69
Baldus *et al.* (2010)[52]	Seq/PyroSeq	20	9 (45)	9[‖] (45)	NR	90
Cejas *et al.* (2009)[53]	Seq	110	37 (34)	40[‡] (36)	SC and MC	94
Cejas *et al.* (2012)[54]	Seq	117	47 (40)	NR	NR	91
Etienne-Grimaldi *et al.* (2008)[55]	PCR-RFLP	48	16 (33)	16[‡] (33)	NR	100
Finkelstein *et al.* (1993)[56]	Seq	23	12 (52)	12[§] (52)	NR	100
Garm Spindler *et al.* (2009)[57]	Seq/qPCR	31	11 (35)	9[‡] (29)	NR	94
Italiano *et al.* (2010)[58]	Seq	62	24 (39)	25[‡] (40)	SC and MC	95
Knijn *et al.* (2011)[59]	Seq	305	108 (35)	104[‡] (34)	SC and MC	96
Losi *et al.* (1992)[60]	AS-PCR	35	25 (71)	25[‖] (71)	MC	100
Loupakis *et al.* (2009)[61]	Seq	43	NR	NR[‡]	NR	95
Mariani *et al.* (2010)[62]	Seq/ARMS	38	20 (53)	19[‡] (50)	SC and MC	97
Melucci *et al.* (2010)[63]	Seq	62	NR	NR	NR	94
Molinari *et al.* (2009)[64]	Seq	37	16 (43)	15[‡] (41)	SC and MC	92
Mostert *et al.* (2013)[65]	AS-PCR/Seq	43	9 (21)	10[‖] (23)	NR	79
Mostert *et al.* (2013)[65]	AS-PCR/Seq	42	9 (21)	5 (12)[¶]	NR	71
Oliveira *et al.* (2007)[66]	SSCP/Seq	28	18 (64)	23[§] (82)	NR	68
Oltedal *et al.* (2011)[67]	PNA-PCR	91[#]	0 (0)	7[§] (8)	NR	92**
Oudejans *et al.* (1991)[68]	ASO	31	15 (48)	17[‡] (55)	NR	87
Park *et al.* (2011)[69]	NR	69	19 (28)	NR	NR	76
Perrone *et al.* (2009)[70]	Seq	29[‡‡]	4/22 (18)	4/17[‡] (24)	SC and MC	80
Santini *et al.* (2008)[71]	Seq	99	38 (38)	36[‡] (36)	SC and MC	96
Schimanski *et al.* (1999)[72]	PCR-RFLP/Seq	32	14 (44)	7[‡] (22)	NR	78**
Thebo *et al.* (2000)[73]	AS-PCR	20[§§]	20 (100)	16[§] (80)	SC	80
Watanabe *et al.* (2011)[74]	AS-PCR/Seq	43	15 (35)	18[‡] (42)	SC and MC	88
Zauber *et al.* (2003)[75]	SSCP	42	22 (52)	22[‡] (52)	SC	100
All	NA	NA	NA	NA	NA	90[‖‖]
BRAF						
Artale *et al.* (2008)[51]	NR	48	2 (4)	1[‡] (2)	SC and MC	98
Baldus *et al.* (2010)[52]	Seq/PyroSeq	55	5 (9)	3[§] (5)	NR	96
Baldus *et al.* (2010)[52]	Seq/PyroSeq	20	1 (5)	1[‖] (5)	NR	100
Cejas *et al.* (2012)[54]	NR	70[#]	1 (1.4)	1[‡] (1.4)	NR	100
Italiano *et al.* (2010)[58]	Seq	57	1 (2)	3[‡] (5)	SC and MC	98
Mostert *et al.* (2013)[65]	AS-PCR/Seq	43	3 (7)	4[‖] (9)	NR	93
Mostert *et al.* (2013)[65]	AS-PCR/Seq	40	2 (5)	1 (3)[¶]	NR	98
Oliveira *et al.* (2007)[66]	SSCP/Seq	28[¶¶]	7 (25)	10[§] (36)	NR	89
Park *et al.* (2011)[69]	NR	71	5 (7)	NR	NR	90
Perrone *et al.* (2009)[70]	Seq	29	2 (7)	1[‡] (3)	SC and MC	91
Santini *et al.* (2010)[76]	NR	208[#]	13 (6)	9[‡] (4)	SC and MC	97
All	NA	NA	NA	NA	NA	96[‖‖]

*As calculated by the authors of this manuscript or within the original publication, unless otherwise noted. [‡]Lymph-node or distant metastases. [§]Lymph node. [‖]Recurrence or distant metastases. [¶]Circulating tumour cells studied. [#]Wild-type *KRAS* population only. **(Over)-estimated using the calculation $1-[(n_{primary}-n_{disseminated})/n_{total}]$. [‡‡]Although 29 patients were included in this study, *KRAS*-mutation status was know for only 22 of the primary tumours and 17 of the disseminated tumours examined in this study. [§§]Mutant *KRAS* population only. [‖‖]Calculated using the equation $[\Sigma(n_{total} \times \text{Concordance rate})]/\Sigma n_{total}$. [¶¶]Microsatellite-stable population only. Abbreviations: ARMS, amplication-refractory mutation system; ASO, allele-specific oligonucleotide hybridization; AS-PCR, allele-specific based polymerase chain reaction; MC, metachronous; NA, not applicable; $n_{disseminated}$, number of patients with KRAS/BRAF-mutated disseminated cells; $n_{primary}$, number of patients with a KRAS/BRAF-mutated primary tumour; NR, not reported; n_{total}, number of patients in the study; PCR-RFLP, restriction fragment length polymorphism PCR; PNA-PCR, peptide nucleic acid clamp PCR; PyroSeq, pyrosequencing; qPCR, quantitative PCR; SC, synchronous; Seq, Sanger-based sequencing using various amplification methods; SSCP, single-strand conformation polymorphism.

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Table 3 | Factors that might affect biomarker concordance during the course of disease

Causal factors	Examples of the effect of the causal factor on biomarkers concordance	References
Clinical and biological		
Biomarker type: genetic vs protein	Difference in detection frequency of the *EML4–ALK* gene rearrangements in NSCLC specimen by IHC, FISH and RT-PCR Difference in detection frequency of *EGFR* gene mutation in NSCLC specimen by IHC, direct sequencing and qPCR	Teixidó *et al.* (2014)[130] Angulo *et al.* (2012)[131]
Biomarker type: radiological vs biological	Difference in evaluation of disease response between AFP level monitoring and RECIST criteria in hepatocellular carcinoma	Personeni *et al.* (2012)[132]
Biological rhythms	Modification in expression of ERGs during the menstrual cycle in ER+ breast cancer	Haynes *et al.* (2013)[133]
Prior neoadjuvant therapy	Difference between post-treatment and pretreatment Ki-67 in breast cancer	Von Minckwitz *et al.* (2013)[134]
Prior adjuvant therapy	Change in ER, PR and/or HER2 status between primary and relapsed tumours in breast cancer	Lindström *et al.* (2012)[34]
Prior interval therapy	Increased incidence of PTEN loss and *PI3K* mutation after anti-HER2 therapy in breast cancer	Chandarlapaty *et al.* (2012)[135]
Logistical and technical		
Tissue origin: distant vs lymph-node metastases	Differences in HER2-evaluation method (IHC and FISH) among primary tumours, lymph-node metastases, and distant metastases in breast cancer	Regitnig *et al.* (2004)[39]
Sampling origin: surgical specimen vs CNB	Discordance in grade, and ER, PR and HER2 status in breast cancer when comparing surgical specimen and CNB	Lorgis *et al.* (2011)[136] Arnedos *et al.* (2009)[137]
Sampling origin: CNB vs FNA	Variation relating to the use of IHC vs ICC for analysis of ER, PR and Ki-67 status in breast cancer	Stalhammar *et al.* (2014)[138]
Tissue and antigen preservation: specimen fixation and conservation	Pre-analytical variables for IHC or FISH analysis of FFPE specimens	Engel *et al.* (2011)[139] Khoury *et al.* (2009)[140]
Tissue and antigen preservation: FFPE vs frozen or fresh	Introduction of mutation artefacts when starting with an old or low abundance DNA sample (demonstrated during assessment of *EGFR* mutations in colon cancer)	Marchetti *et al.* (2006)[141]
Analytical method: specificity and sensitivity	More than 100% circulating-tumour-cell recovery in spike-and-recovery control experiments	Punnoose *et al.* (2010)[142]
Scoring method	Change in Ki-67 evaluation in breast cancer Improve classification of patients likely to benefit from sorafenib using Choi criteria instead of RECIST criteria	Voros *et al.* (2013)[143] Ronot *et al.* (2014)[144]
Laboratory experience: central or reference vs local	*KRAS*, *BRAF*, *NRAS*, *PI3KCA* mutation assessment in *KRAS* wild-type colorectal cancer population HER2 testing (FISH or IHC) in the N9831 breast cancer adjuvant trial	André *et al.* (2013)[126] Perez *et al.* (2006)[117]

Abbreviations: CNB, core needle biopsy; ER, oestrogen receptor; ERG, oestrogen-regulated genes; FFPE, formalin-fixed paraffin-embedded; FNA, fine-needle aspiration; ICC, immunocytochemistry; IHC, immunohistochemistry; NSCLC, non-small-cell lung cancer; PR, progesterone receptor; qPCR, quantitative PCR; RECIST, Response Evaluation Criteria in Solid Tumours; RT-PCR, reverse-transcription PCR; vs, versus.

amplification, magnetics) or droplet digital PCR represents an interesting potential strategy for future therapeutic decision-making.[85] At present, treatment decisions in the metastatic setting are based on analysis of earlier primary tumour samples, or data from emerging techniques such as evaluation of cfDNA or circulating tumour cells (CTCs) for select mutations. Additional studies should be performed to establish whether any concordance between analyses of primary tumours, cfDNA and metastases are clinically relevant for predicting treatment outcome. However, ethical considerations (potential complications and inconvenience to the patient, for example) and costs must be recognized, and will necessarily limit these types of investigations.

Clinical challenges

A validated predictive biomarker can identify patients who are likely to have a favourable clinical outcome —that is, the population with a high response rate or improved survival—after treatment with a specific therapy, hence differentiating responders from non-responders. The low objective response rate for many emerging therapeutic agents and lack of survival benefit with some targeted therapies represent challenges to the validation of biomarkers that could inform treatment decisions. As a result, the predictive biomarkers currently available are validated for only a small percentage of patients with solid tumours (Table 4). A substantial hurdle for biomarker discovery is that agents produced by different pharmaceutical and biotechnology companies are often used in combination regimens, rather than as stand-alone treatments, in order to enhance therapeutic efficacy. Combination therapy obscures the association between any one agent used in the treatment regimen and the biomarkers under consideration. Combination therapies also raise questions about data sharing, collaborations, intellectual property of the integral use of biomarkers (including biomarker analysis methodologies) developed by different companies, and the approaches to validating such biomarkers in clinical trials.

Given that biomarker development is moving oncology toward personalized medicine, the future progress in drug and biomarker research lies in the choice of ideal populations that might benefit from a particular

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Table 4 | FDA-approved targeted agents with demonstrated activity and an effective predictive biomarker of efficacy in solid cancers*

Year of approval	Drug	Clinical biomarker(s)	Target(s)	FDA-approved indication(s)	Patient population positive for biomarker	RR to treatment
1998	Trastuzumab	HER2 overexpression	HER2	HER2-positive mBC: single agent in second-line therapy, and in combination with paclitaxel in first-line treatment	18–20% (HER2-positive population)	15–50%[145,146]
2003	Imatinib	KIT (CD117)	KIT, ABL and PDGFR	In unresectable and/or KIT-positive mGIST	CD117-positive: 95% *KIT*-mutation-positive: 80%	45–83%[147,148]
2004	Cetuximab	EGFR-protein expression‡	EGFR	With irinotecan or as single agent (2007) for EGFR-positive mCRC refractory to irinotecan	60–80%	11–55%[149,150]
2006	Trastuzumab	HER2 overexpression	HER2	With adjuvant treatment for node-positive, HER2-positive BC	18–20% (HER2-positive population)	38% DFS increase[145,151]
2006	Panitumumab	Wild-type§ *KRAS* (specifically at codons 12 or 13 in exon 2)	EGFR	EGFR-expressing mCRC with disease progression on chemotherapy regimens	40–60%	17–58%[92,152]
2007	Lapatinib	HER2 overexpression	HER2; EGFR	In combination with capecitabine in pretreated HER2-positive mBC	18–20% (HER2-positive population)	24–41%[153,154]
2008	Imatinib	*COL1A1–PDGFB* fusion	KIT, ABL and PDGFR	For *COL1A1–PDGFB* gene-fusion-negative metastatic DFSP (or DFSP with unknown mutation status), and as adjuvant therapy in KIT-positive GIST	>95%	36–100%[155,156]
2009	Gefitinib	*EGFR*-activating mutations	EGFR	NSCLC with *EGFR* mutations that respond to or had prior response to gefitinib (limited approval by FDA)	10–15% of white patients and 30–35% of East Asian patients	37–78%[157,158]
2010	Lapatinib	HER2 overexpression	HER2; EGFR	With letrozole in postmenopausal women with hormone-receptor-positive and HER2-positive mBC	18–20% (HER2-positive population)	8–48%[159,160]
2010	Trastuzumab	HER2 overexpression	HER2	With cisplatin and fluoropyrimidine in the first-line treatment of HER2-positive metastatic GC and GEC	7–34%	47%[161]
2011	Crizotinib	*EML4–ALK* translocation	ALK; MET	ALK-positive locally advanced or metastatic NSCLC	1–7%	50–65%[162,163]
2011	Vemurafenib	BRAF V600E mutation	BRAF	Metastatic melanoma with BRAF V600E mutation	80–90% of *BRAF*-mutated population	48–57%[164,165]
2012	Cetuximab	Wild-type§ *KRAS*	EGFR	In combination with FOLFIRI for the first-line treatment of *KRAS*-wild-type patients with EGFR-positive mCRC	40–60%	47–61%[166,167]
2012	Pertuzumab	*HER2* amplification	HER2	In combination with trastuzumab and docetaxel as first-line therapy for HER2-positive mBC	18–20% (HER2-positive population)	24–63%[168,169]
2013	Ado-trastuzumab emtansine	HER2 overexpression	HER2	HER2-positive mBC with prior exposure to trastuzumab and/or a taxane	18–20% (HER2-positive population)	26–64%[170,171]
2013	Afatinib	*EGFR* exon 19 deletions or exon 21 mutation (L858R)	EGFR, HER2 and HER4	First-line treatment of metastatic NSCLC with *EGFR* exon 19 deletions or exon 21 mutations	45% with *EGFR* exon 19 deletion and 41% with *EGFR* exon 21 mutation	56–67%[172,173]
2013	Ceritinib	*ALK* rearrangement	ALK	ALK-positive NSCLC that progressed during or after treatment with crizotinib	2–5%	56%[174,175]
2013	Erlotinib	*EGFR* exon 19 deletion or exon 21 mutation (L858R)	EGFR	First-line treatment of metastatic NSCLC with *EGFR* exon 19 deletions or exon 21 mutations	45% with *EGFR* exon 19 deletion and 41% with *EGFR* exon 21 mutation	54–83%[176,177]
2013	Pertuzumab	*HER2* amplification	HER2	As neoadjuvant treatment with trastuzumab and docetaxel for HER2-positive advanced, inflammatory or early-stage BC	18–20% (HER2-positive population)	24–62%[178,179]
2013	Trametinib	BRAF V600E/K mutations	MEK	Unresectable/metastatic *BRAF*$^{V600E/K}$-mutated melanoma	*BRAF*^{V600E}-mutated: 80–90%; *BRAF*^{V600K}-mutated: 20%	22–25%[180,181]
2014	Dabrafenib	BRAF V600E/K mutations	BRAF	With trametinib for metastatic melanoma with BRAF V600E/K mutations	*BRAF*^{V600E}-mutated: 80–90%; *BRAF*^{V600K}-mutated: 20%	31–76%[180,182,183]

*Data taken from the FDA website[95] on 15th June 2014 and completed using EPAR from the EMA product information.[184] ‡EGFR expression was not confirmed as a predictive biomarker in mCRC. §EMA restricted panitumumab and cetuximab therapy to *KRAS* and *NRAS* wild-type mCRC in 2013. Abbreviations: BC, breast cancer; DFS, disease-free survival; DFSP, dermatofibrosarcoma protuberans; EMA, European Medicine Agency; EPAR, European public assessments reports; FOLFIRI, 5-fluorouracil, folinic acid and irinotecan; GC, gastric cancer; GEC, gastroesophageal cancer; mBC, metastatic breast cancer; mCRC, metastatic colorectal cancer; mGIST, metastatic gastrointestinal stromal tumours; NSCLC, non-small-cell lung cancer; RR, response rate.

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Box 2 | Considerations for procedure standardization

Pre-analytical standardization

- Patient factors: anaesthetic agents; hydration; stress responses; drugs; concomitant diseases or co-morbidities; tissue ischaemia; sample-processing delays (phosphorylation); and other unknown factors
- Tissue factors: collection (device/process, tissue versus serum based specimen, sample volume, contamination); fixation (type, time, penetration); processing (methods, times for each step, temperature); storage; and stability and integrity

Analytical standardization

- Tissue factors: analyte differences (DNA, RNA, protein); antigen retrieval (for immunohistochemistry); antibody variability; detection reagents (chromagens); inconsistencies relating to kits and automation; control selection; and quality control
- Scoring systems for staining: intensity; extent; topography; nonlinearity of methodologies; and computerized image analysis ('precise measurement of the imprecise')

Post-analytical standardization

- Effects of volume of testing by laboratories: high-volume testing laboratories, such as central laboratories, usually have more expertise and proficiency than low-volume local laboratories
- Data interpretation: dichotomous variables; continuous variables (cut-points relevant to clinical decisions); and reproducibility
- Collaborative role of professional pathology organizations: at the international level, to define standards; at the local level, to facilitate implementation of these standards

treatment. However, population stratification in clinical trials narrows the landscape of drug development and, as such, the potential market share for the drug. In fact, the clinical integration of some cancer medications on the market benefited from retrospective biomarker analysis to overcome the difficulties encountered during clinical development, such as limited responses in unselected patients owing to inherent drug resistance. For example, such studies in mCRC identified genetic aberrations that predict outcome of treatment with the anti-EGFR antibodies cetuximab and panitumumab.[86–92] The reduction in the potential market share must be compensated by acceleration of validation and reduction in the cost of drug development. However, the use of selected clinical trial populations raises a challenge for the validation of the biomarker assay itself, as comparison of the outcomes of a potentially biomarker-guided treatment between the biomarker-positive and biomarker-negative populations is ultimately required to assess assay performance. Therefore, although the development of biomarker-based diagnostics is recognized as an important paradigm, technical and economic considerations relating to the standardization of biomarker evaluation and validation must be taken into account.

Logistical challenges

Several ongoing logistical hurdles are linked to integration of biomarkers into clinical trials and the practice of oncology. These include the need for well-managed, centralized specimen biobanks for high-quality biomarker studies and standardization of sample collection, processing, and storage among the facilities, as these factors are critical determinants of the reliability of biomarker analysis. It is clear that statistical analysis of biomarker data is also an important logistical component of the validation process; statistical evaluation is challenging in terms of achieving uniformity in data management, bioinformatics, and biostatistics methodologies.[93] Optimizing outcomes assessment requires multidisciplinary effort and fit-for-purpose statistical methods that rely on a synergy between statistics and biological understanding. Given the inherent methodological challenges of conducting prospective studies to confirm the validity of predictive biomarkers, well-designed retrospective studies, using existing well-characterized samples, can be of great value: such studies can be used to accumulate evidence of biomarker effectiveness more rapidly—albeit lower-level evidence than is provided by prospective studies—and, therefore, support the transfer of candidate biomarkers into clinical practice. However, to yield convincing evidence, so-called retrospective–prospective study designs must be pre-planned (including cut-points and statistical methods) and conducted with reference to standardized guidelines. Moreover, translation of biomarkers from the research laboratory into the real-world setting without loss of analytical performance and standardization is often time-consuming and difficult, as the sensitivity and specificity of biomarkers developed in the research laboratory have to be feasible in the clinical laboratory with regulatory compliance, and meaningful for decision-making in order to guide patient care.[94] Unfortunately, many promising biomarkers fail to meet these requirements and are never used outside of limited applications, such as proof-of-concept testing. Thus, it is crucial that the biomarker validation process is performed in settings mirroring closely the clinical environment.

Considerations for method validation

The global issue for biomarker development is the robustness of the laboratory methodology in all analytical aspects, including assay precision, accuracy, sensitivity, specificity, reproducibility, linearity, reliability, and generalizability. Unfortunately, highly standardized assays for biomarker identification and analysis are rare. In fact, most of the FDA-recognized pharmacogenomic biomarkers[95] are not validated *in vitro* diagnostics (IVDs), but are rather laboratory-developed tests (LDTs). LDTs represent 'in-house' tests that might be subject to considerable interlaboratory variability despite accreditations such as ISO 15189 in the European Union, or Clinical Laboratory Improvement Amendments (CLIA) and Investigational Device Exemption (IDE) in the USA, which are discussed in more detail in a following section. Of note, substantial differences exist in the requirements for accreditation between Europe and the USA.[96]

Biomarker sensitivity and specificity can be interpreted in terms of analytical or clinical performance. Analytical performance must be optimized for three different aspects of the biomarker validation process: pre-analytical, analytical, and post-analytical (Box 2).

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Pre-analytical standardization

Pre-analytical processing is generally considered the greatest challenge in the biomarker standardization process. Indeed, several pre-analytical variables influence the effective assessment of biospecimens, the reliability of the analyses, and the final results of the biomarker evaluation that ultimately influence the patient's care and outcome. These variables include patient factors, such as physiological variables and pathological states, as well as 'specimen and sample factors' that relate to the clinical procedures that are used to obtain the biospecimens (the collection and handling processes), including patient identification; sample labelling or mislabelling; volume of usable material; collection, transport and storage conditions; and processing delays (Box 2).

Guidelines for standardization of samples

To improve standardization of specimens, the US National Cancer Institute (NCI) has published best-practice guidelines for biospecimen resources,[16] as has the International Society for Biological and Environmental Repositories.[97] These documents provide a comprehensive approach to the procedures for tissue collection, processing, banking, retrieval, analysis, and dissemination, as well as issues of ethics, informed consent, privacy, and intellectual property. These reports are oriented predominantly at research use. In the clinical trial setting, reliance on standard pathological material and collection techniques is usually greater than in other research settings, but if the NCI maxims are adopted and applied with rigour, a more-successful biomarkers programme is likely to emerge. In addition, the College of American Pathologists (CAP) has initiated an Accreditation for Biorepositories Program with a clinical perspective.[98] It should be noted, however, that the logistics and cost for achieving better standardization are likely to be burdensome for many institutions, and in some cases might prove to be prohibitively expensive. For instance, 24-hour pathology laboratories could ensure that analyses are performed routinely at the same time interval after samples are obtained, but meeting the cost of establishing such facilities is unrealistic for most centres, given the current constraints on health-care expenditures and research funding.

Important sources of pre-analytical variation

The first step in the pre-analytical standardization of any biomarker assessment is the selection of a meaningful sample that is easy to obtain and optimal for analysis, because sample origin can influence the validation process.[99] In addition, sample and reagent integrity (from sampling through processing), processing conditions, and the elapsed time from sample collection to both processing and analysis can have major impacts on biomarker data. For instance, sample stability is influenced by freezing–thawing, storage duration and temperature, consistency of temperature, and specimen-container types and stabilizers.[100] Time is routinely an influential factor throughout the biospecimen collection and processing period, especially for proteins and peptides that are highly labile and subject to various alterations —in phosphorylation status, for example.[101] By contrast, nucleic acids, in particular DNA, are more stable and, therefore, less sensitive to variation in sample processing times.[102,103] Tissue fixation parameters might also markedly affect the results of biomarker analyses by changing the molecular profile of the analytes:[104] formalin fixation has been shown to substantially reduce DNA and RNA solubility and induce a high frequency of sequence alterations.[105–107] Thus, new methodologies have been developed in attempts to avoid the cumbersome sample freezing process and provide appropriately stabilized fixed tissue with unchanged and well-preserved analytes (DNA, mRNA, and proteins).[108–111]

In addition, patient factors such as the level of hydration, tissue-ischaemia time, stress responses, and concomitant drug and anaesthetic agent effects, as well as heterogeneity of samples that might be composed of normal, tumoural, and/or necrotic tissues, can affect expression of potential biomarkers and their analysis, particularly when samples are obtained during surgery.[112,113] Another important factor is the analyte volumes available for testing: tissue specimens obtain through small biopsies and fine-needle aspiration can limit the analysis. Currently available methods for amplification of material might have utility in overcoming this limitation, but could introduce analysis artefacts.[90]

Addressing pre-analytical variables

Many pre-analytical factors, including those pertaining to the patient as well as others such as the time of day at which an operation is scheduled, cannot realistically be controlled. Therefore, it is important that disease-related and patient-related characteristics (demographics, clinical condition before medical intervention, ischaemic time, and treatment-related variables), and the pre-analytical procedures used are annotated as completely as possible to enable their possible influences on assay results to be considered on a patient-by-patient basis during statistical analysis. Once the sample is obtained from the patient, greater potential for standardization exists. Tissue preparation protocols relating to the timing of fixation, the specific type of fixative and its penetration into tissue, as well as sample processing protocols that outline the timing of each step, procedural temperatures, and subsequent microtomy sectioning and slide mounting of fixed specimens have been addressed.[99] However, despite the rigorous application of protocols, lack of cross-institutional uniformity of procedures remains an issue. For instance, in a comparison of protocols for the pathological examination of prostate cancer needle-biopsy specimens from the 11 institutions enrolled in the NCI Specialized Programs of Research Excellence (SPORE), none of the centres used precisely the same protocol.[114] Lack of uniformity of standards between technology platforms for molecular and pathological analysis has also been an issue recognized by the French National Cancer Institute (INCa) centres.[115] The general lack of standardization is due, in part, to a number of technical limitations, such as differences in performance and

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instrumentation. As an example, all the operational steps required in tissue sample preparation are performed by tissue processing machines, but in adherence with standard operating procedures that are customized locally to account for specific factors, such as the timing of pathological evaluation. Consequently, the procedural steps for which the standard protocols must be re-optimized by laboratories to make them applicable locally can be numerous, leading to substantial variation in the data obtained, which ultimately are not uniform or shareable.

Analytical standardization

The applicability of a qualified biomarker relies on the development of a robust, validated assay with high sensitivity, specificity, precision, and accuracy. Many of the techniques that are currently used in the development of biomarkers for patient stratification, such as immunohistochemistry, FISH or silver *in situ* hybridization (SISH), real-time quantitative reverse-transcription PCR (qRT-PCR), microarrays, epigenetic assays, sequencing, and mutation analyses, continue to lack high-level performance and robust evaluation processes. Development of robust and accurate analytical standards is mostly constrained by tissue availability and the complexity of the biological samples containing DNA, RNA and proteins. For instance, conventional immunohistochemistry, the most widely used platform for biomarker assessment in diagnostic surgical pathology, has been faced with several practical limitations when applied to biomarker examination, such as the selection of the ideal antigen, antibody, detection reagents, kits, and positive and negative controls, and difficulties in quantification with reference standards.

Ensuring reproducibility and concordance

An assessment of accuracy and reproducibility of the diagnostic evaluation of HER2 by immunohistochemistry between two Breast Cancer International Research Group (BCIRG) central laboratories and local laboratories showed an overall concordance of 77.5%;[116] however, a concordance rate as low as 51.7% (281 of 543) was observed for HER2-positive (2+ or 3+) immunostaining patterns.[116] Concordance figures were slightly better in the North Central Cancer Treatment Group (NCCTG) N9831 phase III adjuvant trial, with an overall concordance of 82%.[117] Of note, these data were all derived from CLIA-accredited laboratories. An FDA-cleared kit has been shown to yield similar discordance in HER2 positivity between different laboratories.[118] Such findings indicate that attempts to standardize biomarker methods are essential to ensure uniformity and quality of data collected. Given that laboratory-based evaluation approaches vary worldwide, that pre-analytical standardization is difficult to achieve among centres, and that standardized reagents and analytes are unavailable for most assays, more stringency is clearly needed. For example, an integrated network of high-volume clinical laboratories with proven expertise and proficiency should perform biomarker validation, and establish the baseline for the reference standards proposed by the CAP and ASCO for HER2 testing.[119] Although several aspects of biomarker method standardization have been addressed, and standardized kits and automation have resulted in some marked improvements, immunohistochemical qualitative evaluation and many pivotal analytical procedures, such as fixation and antigen retrieval, remain problematic.[13,15,120] To evaluate the robustness of such evaluations and to enable the clinical application of biomarkers, reference centres could be established to coordinate the activities across centralized laboratories, as has been done in the UK and in Canada.

The lack of sufficient intraplatform and interplatform studies on the concordance of qualitative and quantitative data has also been of major concern for standardization procedures, recognizing that the results for the same biomarker, under similar conditions, could vary substantially among laboratories and across platforms. Given the frequency of biomarker discordance between primary tumours and corresponding metastases assessed by different methods (Tables 1 and 2), questions arise as to which techniques and what concordance levels should be required to ensure consistency. A review of HER2 immunohistochemical test performance among laboratories in patients with invasive breast carcinoma established that an overall ≥90% consensus between all the laboratories, which was achieved for 69% of the samples analysed, was a reasonable indicator of assay performance, even if considerable discordances were observed between the results of tests performed by multiple laboratories using the same standardized equipment and reagents.[118] As illustrated by this study, thresholds for sufficient concordance rates are often arbitrary. It is, therefore, mandatory to collect sufficient data and address this issue further in future attempts to define universally expected concordance rates for biomarkers. The inherent limitations regarding the performance characteristics of laboratory methods (sensitivity, specificity, reproducibility, accuracy, and linearity) make resolution of this problem difficult.

Scoring systems

Scoring is another potential source of variability for which improvements in standardization are required. Despite the widespread immunohistochemical assessment of HER2, oestrogen receptor (ER), and progesterone receptor (PR) expression in routine diagnostic practice, and the availability of antibodies recognizing mutated KRAS and BRAF that enable assessment of *KRAS* and *BRAF* mutation status, no universally accepted scoring standardization for these markers has been realized. Recommended scoring procedures described in immunohistochemistry kits are not always followed closely, leading to decreased reproducibility and sensitivity of the methods; that some laboratory-developed scoring systems might perform better than those recommended by the vendor is also possible. These assays are often evaluated on the basis of archived tissue samples in which storage characteristics can influence protein-expression levels.[121] Moreover, the presence of large numbers of non-neoplastic cell types in

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needle-biopsy specimens can limit the analysis of tumour cells and result in incorrect biomarker evaluation. Finally, immunohistochemical methods are notoriously nonlinear, and scoring systems are generally vulnerable to heterogeneity among intensity, extent, and topography of staining.[12] Unlike mutational analyses, immunohistochemical studies are not dichotomous, which complicates their role in clinical decision-making. Thus, both the proportion and type of cells positive for the targeted antigen as well as both the intensity and pattern of the immunoreactivity should be measured and standardized against reference values.

Computerized image analysis is potentially of value in the scoring of biomarkers. Image analysis can be criticized on philosophical grounds, considering that it provides accurate and precise measurements of data from an inherently imprecise assay method; however, reasonable levels of concordance can be achieved with respect to basic interpretation.[13] A challenge with immunohistochemical scoring of HER2 status was demonstrated in a study in which *HER2* status was evaluated across five laboratories in Europe.[122] Although the laboratories were fully concordant with regard to the interpretation of HER2 status (positive or negative), considerable divergence in scoring (according to the 0 to 3+ scale) was observed, particularly in cases with ambiguous immunochemistry and borderline FISH results.[122]

The influence of technological improvement

Technological improvements generally have a great impact on clinical practice; despite being highly desirable, these improvements can, however, result in confusion regarding clinical decisions. As an example, it has been shown that microfluidic droplet-based PCR technology for the identification of gene mutations has greatly improved the sensitivity of detection for mutations and/or alterations affecting *KRAS*, *BRAF*, and *HER2*.[123–125] The technique enabled the determination and precise quantification of a mutant *KRAS* gene in the presence of a 200,000-fold excess of unmutated *KRAS* DNA (sensitivity of detection of approximately 0.0005%), whereas conventional methods such pyrosequencing or the amplification refractory mutation system (ARMS) gave a sensitivity of detection of approximately 1–10%.[123–125] One study revealed discrepancies in tumour mutation-status assessment by standard methods at local laboratories compared with a central evaluation process.[126] In this study, tissue samples from 60 patients with mCRC that were assessed locally were defined as wild-type *KRAS* codon 12 and codon 13; however, central evaluation showed that 10% of the tumour samples in fact harboured *KRAS* mutated at codon 12, and around 20% displayed rare *KRAS* mutations, or *BRAF* and *NRAS* mutations.[126] Clinicians are thus faced with the question of whether or not the differences in sensitivity, considering analytical and biological variables, represent clinically meaningful information that should influence medical decisions. For instance, should the detection of a low-frequency *KRAS* mutation be a contraindication to anti-EGFR antibody therapy in patients with metastatic colon cancer? To make such decisions easier, widely established cut-off points should be implemented in the interpretation of data on continuous variables, to distinguish meaningful measures that can be transformed into dichotomized decisions: positive versus negative; mutant versus wild type; or eligibility for a treatment or trial versus ineligibility. This type of clinical validation poses a major challenge in clinical research because of the low frequency of patients with such equivocal assay results and, therefore, the large starting population needed to generate a sufficient sample size, as well as the length of follow up needed to complete and analyse trials in order to draw conclusions.

A variety of high-throughput technologies, such as transcriptomic, proteomic, and metabolomic modalities, enable large-scale analysis of complex biological systems to identify candidate biomarkers and characterize relevant pathways. To date, most of these types of analyses have generated valuable research results, pinpointing potential biomarkers for further development. However, the complex workflow of these approaches and the inability to verify some candidate markers in subsequent studies provide evidence that such analyses are, in general, insufficiently robust to be translated into the clinical arena to guide therapeutic choices at present, and intermediate values from such biomarker assay techniques are difficult to interpret.[127] Nevertheless, comprehensive broad-scale assessments that rely on a series of measurements, ideally of different parameters within a multivariate framework, have the potential to provide more-extensive and/or more-robust predictive data, and thus these analytical approaches hold promise in advancement of the current state of the art in clinical practice.

Post-analytical standardization

The post-analytical phase of biomarker evaluations involves reporting of the assay results, including normalization procedures and interpretation. Although thought to be less common than pre-analytical and analytical methodological issues, post-analytical errors, especially those that produce inconsistent values, might affect biomarker performance. Therefore, adequate measures must be taken to ensure a post-analytical phase that is as error-free as possible. Dichotomous variables are relatively straightforward to incorporate into calculations of data sensitivity and specificity. However, most variables in the setting of cell biology are continuous, which raises the problem of consensus with respect to clinically relevant cut-off points for diagnostic testing. This issue was exemplified by the finding that almost 10% of the women with breast cancer included in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 trial had neither *HER2* amplification nor overexpression of HER2 based on centrally reviewed testing for this biomarker, but nevertheless benefited from adjuvant therapy with trastuzumab.[117,128] This result raised question about the current definition of HER2 positivity as an indication for trastuzumab treatment and provided the rationale for the NSABP B-47 trial

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Box 3 | Recommendations for optimizing biomarker evaluation

Preliminary considerations
- Biomarker studies should be based on sound biology and a thorough understanding of the biological relevance of the biomarker and underlying biology
- Consider composite biomarkers panels to improve sensitivity, specificity, and predictive power

Clinical considerations
- Ensure the specimen source (archived tissues, fresh biopsy tissues, metastatic lesions, etc.) most relevant to trial goal, design and ethical standards is used
- Record patient factors and clinical procedural variables that are relevant for each biospecimen
- Perform high-quality correlative studies in clinical trials (use preplanned cut-points and statistical methods with reference to standardized guidelines), obtaining consent for biospecimen-banking to support a wide range of scientific investigation
- Adapt biomarker assays according to the clinical stage of drug development as well as the information that needs to be gathered for both biomarker-assay and drug development: consider using a fit-for-purpose strategy to avoid premature lock-down of biomarker-assay development
- Centralize specimens in a well-managed biobank and biomarker evaluation in core-credentialed laboratories, with a reference centre that will coordinate the activities among the evaluating laboratories
- Ensure rigorous pursuit of defined standards through optimized studies rather than limiting research to clinical data from trials
- Perform biomarker validation in settings mirroring closely the clinical environment

Technical considerations
- Implement best practices for biospecimen resources based on the available guidelines[15]
- Use a specimen source for which easy collection, appropriate volumes and optimal analysis are feasible
- Ensure quality and integrity of biospecimens throughout all processes by using relevant newly developed methodologies
- Concentrate on the specific quality-control and quality-assurance practices for appropriate procurement, formalin fixation, and paraffin embedding
- Consider DNA markers, as these are the most resistant to degradation and alteration, and are more likely to yield a dichotomous end point
- Standardize sample handling—harmonization of collection factors, such as sample labelling, volumes, transport, stabilization and storage—and processing methodology, including delays and data collection/annotation
- Ensure fit-for-purpose approaches
- Develop procedures with rigorous quality assurance, reproducibility, and control procedures built-in
- Collect data on the effects of methodological variables on assay performance to construct calibrators and control materials for routine real-world consensus performance
- Pre-define a threshold (cut-off point) for designating the status of the potential biomarker
- Consider using computerized image analysis

Logistical considerations
- Foster collaboration between professional medical associations, investigators, clinicians and statisticians for their diverse and valuable inputs in assay standardization and validation
- Use fit-for-purpose statistical methods that rely on a synergy between statistics and biological understanding

that is currently investigating whether women with 1+ ('HER2-negative') and non-*HER2*-amplified 2+ (HER2-low) breast cancers benefit from addition of trastuzumab to adjuvant chemotherapy.[129] Data based on continuous variables also tend to be less reproducible than information on dichotomous parameters. Thus, successful standardization of post-analytical biomarker methods in this setting requires close collaboration between professional medical associations, investigators, clinicians, and statisticians. In fact, so-called 'dichotomous' variables have been made dichotomous because a cut-off point has been established either actively, as was the case for gene-copy numbers in FISH analysis of *HER2*, or passively enforced by technical limitations such as sensitivity of qRT-PCR and sequencing in the detection of mutations.

Recommendations for biomarker studies

One should not be dismayed at the long list of varied challenges to biomarker method development, and instead thoughtfully acknowledge and address these issues to drive continuous improvements, as rigorous assay validation is expensive in terms of time, materials, financial costs, and biological specimens. Depending on the complexity and the intended purpose of the biomarker, its development will take several years and costs might rise to over US$100 million, for companion biomarkers in particular, owing to the requirement for large retrospective studies and prospective validation trials. As biomarker measurement and standardization can be assessed at several levels, different types of considerations should be addressed in order to maximize successful biomarker evaluation. Examples of such preliminary theoretical, clinical, technical, and logistical considerations are provided in Box 3. Ultimately, data should support the cost–utility of biomarker methodologies to ensure cost-effective clinical decision-making.

Conclusions

As biomarkers have increasingly important roles in drug development and clinical trials, quality assurance and method validation have become crucial, and highlight the necessity of establishing standardized methodological guidelines. The ultimate goal for a biomarker is the establishment of clinical utility that guides patient care, but attempts to reach this goal must be preceded by analytical and clinical validation of the 'locked-down' biomarker assay. Substantial progress has been made in biomarker research, from discovery to development, standardization, and clinical application. However, major challenges regarding integrated and harmonized processes, spanning pre-analytical, analytical and post-analytical phases of development, remain. In the era of targeted therapies, the need for standardized approaches for biomarker validation has become widely recognized as an important issue to overcome. Several joint collaborative initiatives across different sectors in the USA and worldwide have emerged to address the lack of standardized guidelines in biomarker validation, specifically regarding biological specimens and assay methodologies. Although these efforts have contributed to the promotion of standardized procedures, sustained and continued commitment to ensure worldwide standards and harmonization are required.

It is important to recognize the fact that, even if all of the above recommendations are addressed, several additional factors will continue to pose major challenges: the complexity of the biological systems under investigation; the marginal effects provided by many drugs; the continuous nature of the data from assays

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for many potential biomarkers; and the inevitable variability among patients. Therefore, that basic assessment methodologies are robustly qualified, and are applied with rigorous adherence to high methodological standards and close attention to guidelines at each successive step of the validation process, including pre-study and in-study method validation, is essential if we are to obtain reliably validated biomarkers for routine use. Investment of effort and resources in the development of these biomarkers will expand their roles as valid end points for assessing patient outcome.

Review criteria

PubMed, Google Scholar, www.fda.gov, www.ema.europa.eu and/or Google were searched for English-language, full-text manuscripts, abstracts or posters using the following search terms alone and in various combinations: "predictive biomarker"; "biomarker development"; "biomarker standardization"; "biomarker economics"; "biomarker concordance"; "biomarker challenges"; "HER2"; "KRAS"; "BRAF"; "breast cancer"; and "colon cancer". The reference lists of the articles identified were also searched for additional relevant publications.

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