



## Laboratory-Clinic Interface

## Strategies to design clinical studies to identify predictive biomarkers in cancer research



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## ABSTRACT

The discovery of reliable biomarkers to predict efficacy and toxicity of anticancer drugs remains one of the key challenges in cancer research. Despite its relevance, no efficient study designs to identify promising candidate biomarkers have been established. This has led to the proliferation of a myriad of exploratory studies using dissimilar strategies, most of which fail to identify any promising targets and are seldom validated. The lack of a proper methodology also determines that many anti-cancer drugs are developed below their potential, due to failure to identify predictive biomarkers. While some drugs will be systematically administered to many patients who will not benefit from them, leading to unnecessary toxicities and costs, others will never reach registration due to our inability to identify the specific patient population in which they are active. Despite these drawbacks, a limited number of outstanding predictive biomarkers have been successfully identified and validated, and have changed the standard practice of

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oncology. In this manuscript, a multidisciplinary panel reviews how those key biomarkers were identified and, based on those experiences, proposes a methodological framework—the DESIGN guidelines—to standardize the clinical design of biomarker identification studies and to develop future research in this pivotal field.

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## Introduction

Even though personalized oncology is widely perceived as an imminent reality, few anticancer drugs are currently prescribed based upon predictive biomarkers [1]. Moreover, despite outstanding advances in molecular biology, the clinical development of most anticancer drugs is still based on conventional randomized studies that aim to detect statistically significant clinical benefits in unselected patients. Several factors underlie this fact, including the complexity of cancer, but probably one major cause is the lack of a robust methodology to discover candidate biomarkers. Indeed, much attention has been paid to biomarker validation [2–10], certainly a critical step in biomarker development. Yet, these resource and time-consuming procedures cannot be applied to every candidate. Therefore, in analogy with classical drug development, where phase I and II trials select which candidates should undergo further testing, a well-defined methodology is required to identify the most promising candidate biomarkers that should advance towards validation.

The absence of such a methodology has relevant implications for cancer research, including the proliferation of exploratory studies that fail to identify robust candidates; the inability to compare biomarkers across studies and to select the most reliable results; and foremost, the absence of solid biomarker identification programs in the clinical development of many anti-cancer drugs. This results in many patients receiving drugs that will not benefit them; whereas some drugs that may be effective for some specific patients will never be registered, due to our inability to identify such target populations.

The lack of biomarkers reduces the efficacy of many anti-cancer drugs, ranging from cytotoxic chemotherapy to antiangiogenics. Yet, since predictive biomarkers represent the functional presence or absence of the molecular mechanisms of action and resistance characteristic of each drug, it seems reasonable to hypothesize that all drugs should have predictive biomarkers, and that these might be identified using appropriate strategies. Moreover, discovery of biomarkers allows to understand such mechanisms of sensitivity and resistance, and to develop improved therapeutic strategies to overcome resistance, such as the combination of *MEK* inhibitors and *BRAF* inhibitors for melanoma, [11] or the design of new generation *EGFR* [12] or *ALK* inhibitors [13] for lung cancer. On the contrary, the lack of biomarkers hampers such developments, as well as the validation of known biomarkers in different tumor types.

Despite these drawbacks, some outstanding biomarkers have been successfully incorporated into standard oncology practice, transforming drugs with limited efficacy in unselected patients into core elements of our therapeutic arsenal. This manuscript revisits how these biomarkers were identified and draws upon these successful experiences to propose a methodological framework, the DESIGN guidelines, to standardize and expand this pivotal field.

## Methods

Using the published literature [14] and open-access internet resources [15] we identified predictive biomarkers that are routinely used to prescribe targeted drugs for patients with solid

tumors. We reviewed how each biomarker was identified from a clinical and preclinical standpoint (Table 1). The results were analyzed by a panel formed by experienced specialists in biomarker research from several fields, including: medical and radiation oncology, pathology, molecular oncology, cancer immunology, cancer genetics, clinical biochemistry, research nursing, research ethics and biostatistics. The panel also discussed how the regulatory and ethical environments could further support biomarker development.

## Results

We identified 8 predictive biomarkers that drive prescription of targeted drugs for solid tumors in standard practice (Table 1):

### Hormone receptors

Beatson established the basis for the hormonal treatment of breast cancer in 1896, confirming the activity of oophorectomy in this disease [16]. His seminal report was based on the effects of ovarian castration on mammary glands of farm animals and, quite amazingly, he pioneered modern translational research by over a century, by performing for the first time sequential tumor biopsies in patients before and after treatment.

Later on, pharmacological hormonal inhibitors were developed, but their relatively low activity in unselected breast cancer patients led to decreased interest in this approach [17], in favor of chemotherapy. Jensen observed that tritium-labeled estradiol-17, 3 injected in immature rats was preferentially bound in the uterus [18], leading to the identification of estrogen receptors (ER) [19]. Subsequently ER tumor levels were correlated with clinical activity in retrospective analyses of 33 patients undergoing endocrine therapy for advanced breast cancer [20] and transformed a maneuver of moderate efficacy into one of the most relevant therapeutic strategies in the history of oncology.

### HER2 overexpression

*HER2* (Receptor tyrosine-protein kinase *erbB-2*) was identified in 1981 [21] and was found to be markedly amplified in breast cancer cell-lines [22]. Expression of activated *HER2* in transgenic mouse models induced malignant transformation of breast epithelial cells [23]. *HER2* overexpression was observed in 15–20% of breast cancers, conferring a poor prognosis [24], and anti-*HER2* mouse monoclonal antibodies inhibited breast cancer proliferation *in vitro* [25].

Trastuzumab, a humanized anti-*HER2* antibody, showed activity in breast cancer patients overexpressing *HER2* [26]. Phase III studies confirmed that trastuzumab and chemotherapy prolonged respectively overall survival (OS) and progression-free survival (PFS) in *HER2+* breast cancer patients in advanced [27] and adjuvant settings [28], as well as OS in patients with *HER2+* advanced gastric cancer [29]. *HER2* overexpression also predicts efficacy of *HER2* tyrosine-kinase inhibitors (TKI), such as lapatinib or neratinib and of newer monoclonal antibodies targeting *HER2*, such as pertuzumab [30] or trastuzumab-DM1 [31].

**Table 1**  
Methodological characteristics of studies that identified predictive biomarkers that are routinely used in clinical practice for solid tumors.

Biomarker	Studies	Design	Single agent	Disease setting	Study endpoint	Phenotype selection	Sample size	Type of sample	Molecular nature of the biomarker	Preclinical evidence	Validation	Biomarker expression in peripheral blood	Interval between target description and discovery of biomarker (years) <sup>a</sup>
Hormone receptors	Jensen [20]	Retrospective	Yes	Advanced	Response rate	No	33	Fresh or frozen tumor biopsy	Overexpression of tumor receptor	Yes, before clinical evidence [19]	NCI Consensus [180]	Yes [181]	75
Her-2	Baselga [26]	Prospective, phase II	Yes	Advanced	Response rate	No	46	Paraffin embedded tumor biopsy	Overexpression of tumor receptor	Yes, before clinical evidence [25]	Phase III study [27]	Yes [182,183]	15
C-KIT	Joensuu[37]	Prospective, single patient	Yes	Advanced	Response rate	No	1	Paraffin embedded tumor biopsy	Mutation in tumor receptor kinase domain	Yes, before clinical evidence [36]	Phase II study [38]	Yes [184]	15
EGFR	Paez [52] Lynch [53]	Retrospective	Yes	Advanced	Response rate	Yes	9/16	Frozen and paraffin embedded tumor biopsy	Mutations in tumor receptor kinase domain	Yes, after clinical evidence [52,53]	Retrospective [54,55] and phase III studies [56–58]	Yes [185]	24
ALK	Kwak [61,62]	Prospective, phase I	Yes	Advanced	Response rate	Yes	2/37 <sup>b</sup>	Paraffin embedded tumor biopsy	Translocation in tumor receptor gene	Yes, before clinical evidence [59,60]	Phase II [62], retrospective [64] and phase III studies [65,66]	Yes [186]	2
KRAS	Lievre [78]	Retrospective	No	Advanced	Response rate, OS	No	30	Frozen tumor biopsy	Mutation in tumor protein	Yes, after clinical evidence [79]	Retrospective studies [80–83]	Yes [187]	26
BRAF	Flaherty (escalation phase) [94]	Prospective, phase I	Yes	Advanced	Response rate	No	55	Paraffin embedded tumor biopsy	Mutation in tumor receptor kinase domain	Yes, before clinical evidence [93]	Phase I (expansion phase) [94] and phase III studies [95]	Yes [188]	22
BRCA	Fong [104]	Prospective, phase I	Yes	Advanced	Response rate	No	60	Peripheral blood	Germline mutation	Yes, before clinical evidence [102,103]	Retrospective, preplanned[105]	Yes (germline)	25

Abbreviations: EGFR: Epidermal Growth Factor Receptor. NSCLC: Non-small cell lung cancer. OS: Overall survival. PFS: progression-free survival.

<sup>a</sup> From description of target relevance in cancer to identification of the biomarker.

<sup>b</sup> The study was performed in 37 patients with solid tumors, but ALK translocations were identified in 2 NSCLC patients that developed a partial response.

### *c-KIT mutations*

Gastrointestinal stromal tumors (GIST) express the stem cell factor receptor *c-KIT*, described in 1986 [32]. Activating *c-KIT* mutations, initially reported in haematological malignancies [33], were subsequently discovered in 80–90% of GISTs [34]. Imatinib, a TKI that targets oncogenic forms of *ABL*, *PDGFR* and *c-KIT* [35], showed anti-tumor effects against GIST *c-KIT* mutated cell lines [36]. Impressive activity of imatinib in one GIST patient [37] was subsequently confirmed in 147 patients [38]. *PDGFR* activating mutations also predict response to imatinib in GIST [39] and in other diseases, such as dermatofibrosarcoma protuberans [40].

### *Epidermal growth factor receptor (EGFR) mutations*

Cohen et al. described *EGF* in 1962 [41] and purified its receptor, *EGFR*, in 1980 [42]. Both molecules were involved in an autocrine loop that promoted tumor proliferation and survival [43]. The *EGFR* TKIs erlotinib and gefitinib induced G1 arrest of cancer cell-lines that expressed *EGFR* [44,45]. Clinical activity in non-small cell lung cancer (NSCLC) was observed since the first phase II trials [46,47] and led to four phase III trials comparing two standard platinum-based regimens with or without each *EGFR* inhibitor [48–51], which to the great surprise of the oncology community were all negative.

Enthusiasm was rekindled when *EGFR* mutations were shown to predict efficacy of *EGFR* TKIs [52,53]. Strikingly, this seminal finding was reported in two studies including just 9 and 16 patients respectively, in sharp contrast with the more than 4000 patients enrolled in the phase III trials. Both groups sequenced *EGFR* exons in human NSCLC samples and identified mutations in exons 18–21. Such mutations were observed respectively in 8 of 9 responders and in none of the 7 gefitinib-resistant patients [53]; and in all 5 responders and none of the 4 resistant patients [52]. Both reports also confirmed that *EGFR* mutations predicted activity of gefitinib in NSCLC cell-lines. These results were prospectively validated in non-randomized [54,55] and randomized trials [56–58].

### *ALK translocations*

In 2007 Soda et al. described a fusion between echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma-kinase (*ALK*) genes by generating a retroviral cDNA expression library from a lung adenocarcinoma specimen surgically resected from one patient [59]. The fusion gene drove neoplastic transformation and chemical interference with *ALK* kinase induced cell death. Crizotinib, a dual *MET/ALK* kinase inhibitor, was found to inhibit *ALK*-mediated signalling in cell lines presenting *ALK* rearrangements [60]. The first phase I trial with crizotinib confirmed striking activity in two NSCLC patients harboring *ALK* gene rearrangements [61,62]. Crizotinib showed a 57% response rate in a cohort of 82 NSCLC patients presenting *ALK* rearrangements, identified by screening 1500 patients [62]. This prompted two phase III trials comparing crizotinib with standard first and second-line chemotherapy in patients presenting *ALK* rearrangements. Nevertheless, the overwhelming results of the initial trial and of a confirmatory study [63], as well as retrospective data confirming that *ALK* rearrangements were not a favourable prognostic factor for NSCLC untreated with crizotinib [64], led to the accelerated approval of crizotinib before registration trials were reported [65,66]. *ALK* translocations also predict efficacy of crizotinib in other tumors, such as *ALK*-rearranged inflammatory myofibroblastic tumor [67] or *ALK* positive lymphoma [68,69].

The inhibitory activity of crizotinib on the growth of the *ROS1*-rearranged NSCLC line HCC78 prompted treatment with

crizotinib of a NSCLC patient harboring *ROS1* rearrangements, who responded to therapy [70]. A confirmatory trial performed in 50 NSCLC patients harboring *ROS1* rearrangements led to regulatory approval of crizotinib for this patient population [71].

### *RAS mutations*

*RAS* proteins comprise a family of ubiquitously expressed GTPases involved in cell proliferation/differentiation and include *KRAS*, *NRAS* and *HRAS*. *RAS* acts downstream of the *EGFR* receptor-signaling pathway. Sato et al. developed 225 IgG1, a murine monoclonal antibody that inhibited *EGFR* with comparable affinity to the natural ligand [72] and induced receptor dimerization and internalization, a relevant mechanism for target inhibition [73]. Cetuximab, a chimeric human:mouse version of 225, showed higher affinity for *EGFR* [74] and demonstrated activity in patients with colorectal cancer expressing *EGFR* [75]. Randomized studies confirmed improved PFS and response rate of cetuximab [76] and panitumumab [77], a fully human IgG2 anti-*EGFR* monoclonal antibody, leading to regulatory approval.

In 2006, Lievre et al. retrospectively evaluated *KRAS*, *BRAF* and *PIK3CA* tumor mutations and *EGFR* copy number in 30 colorectal cancer patients treated with cetuximab [78]. *KRAS* mutations were described in 13 tumors (43%) and they were significantly associated with lack of response to cetuximab (0% mutations in 11 responders vs. 68% in 19 non-responders,  $p = 0.0003$ ) and with OS (16.3 vs. 6.9 months,  $p = 0.016$ ). Transfection of the mutant *KRAS* allele (Gly12Val) to colorectal cancer cell lines rendered them resistant to cetuximab [79]. Retrospective validation was performed in independent series [80,81] and in the cetuximab and panitumumab registration trials [82,83], leading to approval of both agents for *KRAS* wild-type advanced colorectal cancer. Subsequently, *NRAS* and additional downstream mutations, have been associated in retrospective analyses with lack of response to cetuximab [84,85].

### *BRAF mutations*

In 1983 Rapp et al. cloned *c-RAF*, the cellular homologue of the *v-RAF* oncogene [86], and two related genes (*ARAF* and *BRAF*) were described in vertebrates [87]. *RAF* is a family of serine-threonine kinases which act mainly on the *RAS/RAF/MEK/ERK* pathway. *ERK* hyper-activation had been described in melanoma cell-lines [88] and activating *BRAF* mutations were observed in 40–60% of melanomas [89]. The most frequent mutation is the substitution of valine by glutamic acid at position 600 (p.V600E) [89,90], which locks the kinase domain into an active conformation that renders it 480-fold more active than wild-type *BRAF* [91]. Mice models with restricted transgenic expression of mutated *BRAF* in melanocytes confirmed its oncogenic role [92].

Vemurafenib selectively blocks the active kinase, inhibits *ERK* phosphorylation, induces cell-cycle arrest and apoptosis in *BRAF*<sup>V600E</sup> bearing tumor cell-lines and induces regressions of *BRAF* mutated tumor xenograft models [93]. A phase I study with vemurafenib found a 69% response rate in 16 melanoma patients harboring V600E mutations, while none of the 5 wild-type patients responded [94]. A phase III trial confirmed that vemurafenib improves survival in patients with advanced melanoma expressing V600E mutations, as compared with dacarbazine [95]. *BRAF* V600E mutations also predict the activity of *BRAF* inhibitors in other tumor types [96].

### *BRCA mutations*

In 1990, the 17q21 chromosome region was linked to inherited breast cancer susceptibility, through the study of 23 families



comprising 146 cases of breast cancer presenting familial aggregation and including a high number of early-onset and bilateral tumors and male patients [97]. In 1994, *BRCA1* was identified, along with predisposing germinal mutations [98]. Simultaneously, *BRCA2* was mapped to chromosome 13q12–q13 [99] and the gene and predisposing mutations were also identified [100]. Both genes played critical roles in DNA repair, cell cycle checkpoint control, and maintenance of genomic stability, and thereafter, recommendations for cancer surveillance and risk reduction for individuals carrying mutations in the *BRCA1* or *BRCA2* genes were proposed [101].

While normal cells from affected individuals carry heterozygous loss-of-function *BRCA* mutations, inactivation of the remaining wild-type allele is required to drive carcinogenesis. This renders tumor cells more sensitive than normal cells to blockade of DNA repair pathways, such as poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) [102,103].

A phase I study of the PARP inhibitor olaparib, in 60 patients with advanced ovarian cancer showed activity in 12 of 22 patients carrying *BRCA1/2* mutations (54%) [104]. A randomized phase II study comparing maintenance olaparib versus placebo in platinum-sensitive ovarian cancer patients confirmed improved PFS from 4.3 to 11.2 months in patients harboring *BRCA1/2* mutations, in a planned retrospective analysis [105].

## Analysis of methodological aspects of biomarker identification studies

### Related to the trial design

#### Prospective vs retrospective design

Remarkably, despite the theoretical inferiority of retrospective versus prospective studies, three biomarkers were identified retrospectively (Table 1) [20,52,53,78], and moreover, in the setting of conventional treatments, rather than in clinical trials. This challenges the traditional concerns about retrospective studies, and indicates that whenever the quality of the samples and of the clinical data is adequate, they represent a useful tool to identify predictive biomarkers. Advantages of prospective designs include the possibility of studying biomarkers for drugs under development and the optimization of sample collection, whereas retrospective designs take advantage of the large number of patients that receive standard treatments and of the availability of clinical follow-up. Possibly, an adequate strategy is to collect samples prospectively, from patients treated in clinical trials as well as in standard care, and to study them retrospectively, once the sample size, follow-up and working hypothesis make it appropriate.

#### Single-agent vs combination therapy

All the biomarkers, except *KRAS* mutations, [78] were identified in monotherapy studies (Table 1). Single-agent studies seem more adequate to identify candidate biomarkers, since they eliminate the interactions of the combined drugs. Even though this statement might seem obvious, many widely studied drugs considered as targeted agents, such as bevacizumab, have rarely been explored in monotherapy [106,107], and never in the setting of biomarker identification studies.

Since combinations are fundamental in cancer therapy, it seems logical to validate biomarkers identified for single-agents in patients treated with combinations that include such drugs; and to take advantage of the opportunity that combinations represent to identify novel biomarkers (e.g.: studying patients that respond to the combination in the absence of predictive biomarkers of sensitivity to the single-agent; or despite the presence of biomarkers of resistance to the single-agent).

#### Disease setting

All the biomarkers reviewed were identified in patients with advanced disease (Table 1). This setting provides greater opportunities than adjuvant therapy, because drugs are explored earlier in advanced tumors and single-agent therapy is more frequently administered. Also, treatment outcomes in advanced disease rely mainly on drug activity, rather than on staging or efficacy of local treatment, as it happens in the adjuvant setting. It also allows access to sequential biopsies and to use tumor response as an endpoint.

The neoadjuvant setting also allows access to sequential tumor samples and exploration of tumor response, although patients may only receive short courses of therapy, in order to avoid delaying surgery excessively. Also, identification of a biomarker in a specific setting does not allow to extrapolate it automatically to other situations.

#### Clinical efficacy endpoints

Most of the studies used response rate as the endpoint, with the exception of the study that identified *KRAS* mutations (response rate and OS) [78] (Table 1).

Response rate is an adequate endpoint for biomarker identification studies, since it correlates with either the presence or the absence of a direct effect of the drug on tumor growth. Response and also PFS rely directly on the efficacy of the drug, independently of subsequent treatments, and can be evaluated in relatively short periods. Yet, the limitations of response for targeted [108] and immunomodulatory drugs [109]; and the lack of clear effects of some immunotherapies on response and PFS, despite increases in OS, should be considered [110]. OS is usually preferred for biomarker validation, although the effects of treatment cross-over, required for ethical reasons when highly active drugs are studied, must also be considered.

Validated tumor burden-related serum biomarkers (e.g., PSA) and metabolic imaging, (e.g., PET scans) might serve as surrogates of response. Other relevant endpoints (e.g., cardiac toxicity, etc.) should be characterized by the appropriate specific tests. Well-characterized pharmacokinetic [111,112] and pharmacodynamic variables (e.g., ERK pathway inhibition in melanoma) [94] may also be used as primary or secondary end-points for biomarker identification studies.

#### Phenotype selection

While some studies were performed in unselected patients [20,26,37,78,94,104], others selected patients presenting phenotypes of marked sensitivity or resistance to the treatment studied [52,53,62], leading to dramatic decreases in the required sample size (Table 1).

Recently, some studies have identified potential predictive cancer biomarkers even in a single patient, following the strategy of extreme phenotype selection (Table 2). Moreover, the US National Cancer Institute (NCI) has created a specific research program, the Exceptional Responders Initiative [113] and sponsors a clinical trial based on this strategy (NCT02243592).

Extreme phenotype selection has underpinned some outstanding discoveries in oncology, as reviewed elsewhere [114,115], and has proved useful to interpret the large amount of data generated by high-throughput techniques [116–120]. It reduces the sample size required for molecular studies because it enriches biomarker expression in the patients studied and excludes from the analysis patients with intermediate phenotypes of uncertain significance that might confound the information provided by unequivocal phenotypes.

Extreme phenotypes may be defined as patients that present clear responses or progressions with the drug studied; remarkably long or short OS or PFS intervals (e.g., below the 10th, or over the 90th percentile); combinations of either criteria; or marked

**Table 2**

Selected cancer predictive biomarkers identified using selection of extreme phenotypes.

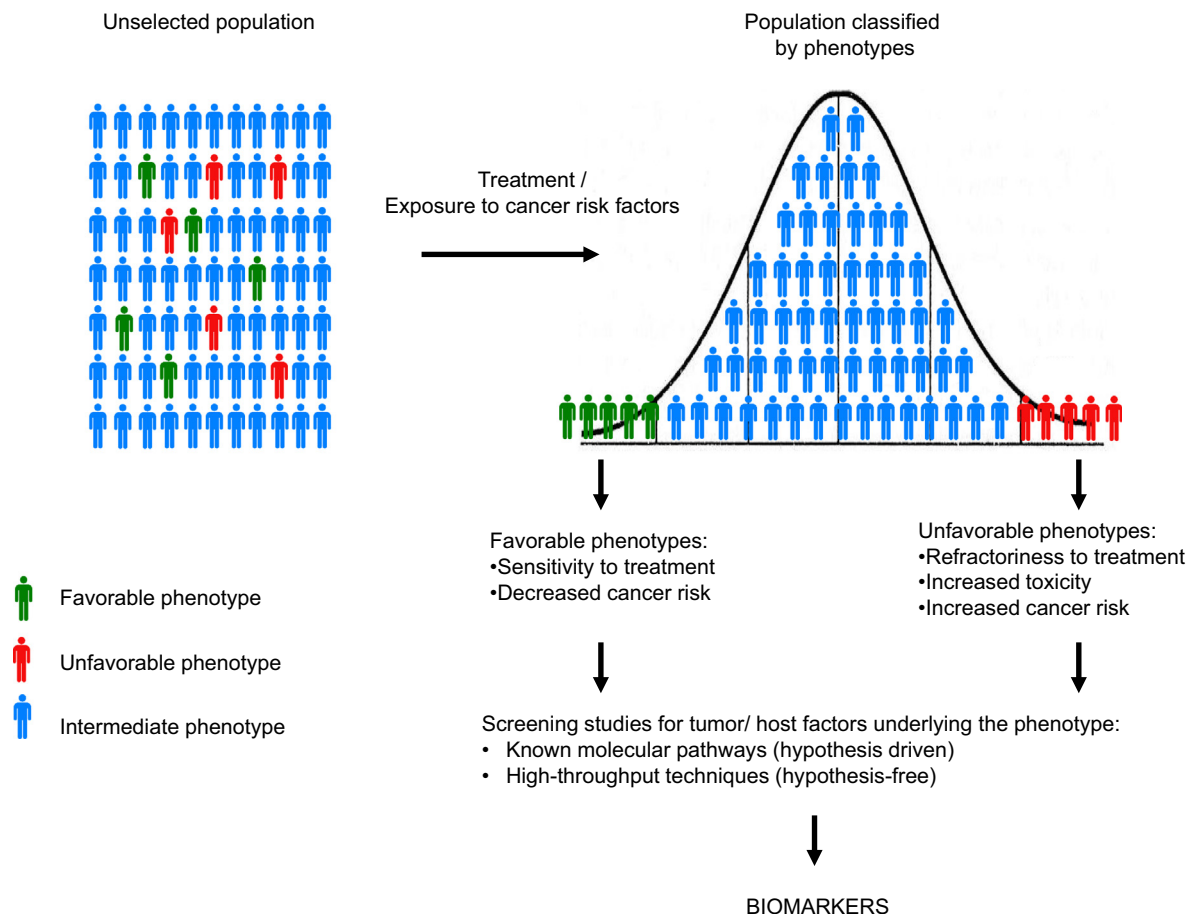
Reference	Tumor	Drug	<i>n</i>	Biomarker	Predictive effect of the biomarker	
Tuchman, [121]	Van Kuilenburg [122]	Colorectal cancer	5-fluorouracil	1	<i>DPD</i> polymorphisms	Marked 5- fluorouracil induced toxicity
Ando [123]	Colorectal cancer	Irinotecan	26	<i>UGT1A1</i> polymorphisms	Severe toxicity with irinotecan	
Iyer [117]	Advanced urothelial carcinoma	Everolimus	1	Inactivating <i>TSC1</i> mutation	Complete response ( > 24 m)	
Wagle [118]	Anaplastic thyroid cancer	Everolimus	1	Inactivating <i>TSC2</i> mutation	Partial response (18 m)	
Wagle [119]	Advanced urothelial carcinoma	Everolimus	1	Activating <i>mTOR</i> mutations	Complete response (14 m)	
Van Allen [120]	Muscle invasive urothelial carcinoma	Cisplatin	25 responders / 25 non-responders	<i>ERCC2</i> somatic mutations	Correlation with complete pathologic response	
Doebele [189]	Soft tissue sarcoma	LOXO-101	1	<i>LMNA-NTRK1</i> fusion	Major partial response (4+ m)	
Van Allen [190]	Head and neck cancer	Erlotinib	1	<i>MAPK1 E322K</i> mutation	Complete response in the neoadjuvant setting	
Cools [191]	Hypereosinophilic syndrome	Imatinib	11	<i>FIP1L1-PDGFR</i> fusion	Correlation with clinical response	

DPD: dihydropyrimidine dehydrogenase. UGT1A: UDP-glucuronosyltransferase 1A1.

toxicity [121–123] (Fig. 1). This strategy may also be used to identify biomarkers associated with increased or decreased cancer risk [114,124,125]. Indeed, extreme phenotypes constitute real-life clinical models of sensitivity and resistance to carcinogens and drugs that may be used to study the underlying molecular mechanisms. Patients that are resistant to a given treatment, despite expression of biomarkers of sensitivity (e.g., NSCLC patients

expressing EGFR mutations who do not respond to EGFR TKIs) or viceversa, also represent extreme phenotypes worth of evaluation.

Additional research is needed to further develop this strategy, including the optimal definition of extreme phenotypes, the determination of the sample sizes, or the extrapolation of the results to general patient populations (e.g., extreme phenotype vs. control population designs) [126].



**Fig. 1.** Design of biomarker identification studies using selection of extreme phenotypes. Exposure of an unselected population to a treatment or risk factor will reveal individuals presenting favorable or unfavorable extreme phenotypes. Since it is not possible to anticipate which patients will present extreme phenotypes, this strategy requires samples to be obtained from all patients, although only extreme cases will be studied. Study of samples may be directed towards specific pathways (hypothesis-driven) or performed with high-throughput techniques (hypothesis-free).

### Sample size

None of the studies reviewed included an approach to determine the sample size for biomarker identification, as in fact occurs in most biomarker identification studies. This calculation is jeopardized by the fact that the determining factors (i.e., nature, frequency of expression, capacity of prediction of the biomarker, sensitivity and reproducibility of the techniques, etc.) are uncertain. In addition, studies that use high-throughput techniques are hypothesis-free, because they lack an alternative hypothesis regarding which factors will be predictive [127]. Finally, the sample size is limited in real life by the scarcity of quality samples and by the capacity and costs of molecular techniques.

The “omics” technologies (genomics, transcriptomics, proteomics, etc.) have been applied to identify biomarkers in a high-throughput manner [128]. In these experiments the number of parameters measured (hundreds or thousands) vastly exceeds the number of subjects included in the study, and thus many standard statistical methods are not applicable. This includes some of the most widely used algorithms to estimate sample size and statistical power for detecting biomarkers [129,130]. Indeed, some calculation methods for specific technologies have been developed [131,132], but their validity is limited by the factors mentioned above and their inability to keep up with the constant improvement in molecular biology techniques.

Since sample size determination is of paramount importance, and until such limitations are overcome, empirical approaches may be pursued. This strategy is already used for phase I studies, which include 3–6 patients per dose level, figures based on no compelling scientific evidence [133]. The sample size range of the studies reviewed (1 [37] to 60 patients [104]) may guide empirical determination of sample size and suggests that exceedingly large samples are not required to successfully identify biomarkers. Even though the use of very reduced samples (e.g., <5 patients) may not be optimal, prior experiences indicate that they may be sufficient, whenever the preclinical evidence for the biomarker is straightforward [37,61] and/or when selection of extreme phenotypes is used [52,53]. On the other hand, the use of larger sample sizes should provide increased statistical power; or the possibility to identify additional biomarkers with a similar biological effect.

### Biological samples

Tumor tissue constitutes the cornerstone for biomarker identification, and was used to identify all the biomarkers reviewed, except *BRCA1/2* mutations [104] (Table 1). Nevertheless, its limited availability represents a major barrier for biomarker research and establishes a vicious cycle in which the absence of adequate samples hampers the development of reliable biomarkers, which consequently questions the rationale for obtaining biopsies.

Three biomarkers were identified in formalin-fixed paraffin-embedded (FFPE) tissue [26,37,61,94] two in fresh or frozen tumor [20,78] one in both FFPE and frozen tumor [52,53] and one in peripheral blood [104]. Even though FFPE tissue is universally available, its efficiency for high-throughput nucleic acid analyses is compromised by the size of the DNA/RNA fragments obtained, the presence of multiple potential inhibitors of reactions, the false positive transitions and other artifacts related to the fixation process in amplicon-based massively parallel sequencing (e.g., Next Generation Sequencing, NGS). Even though technical developments and bioinformatics allow the use of FFPE tissue for NGS-based clinical tests and research, frozen tissue is still more appropriate for this purpose. Therefore, routine acquisition of both frozen and FFPE tumor tissue is paramount to develop solid biomarker research programs. Tumor microenvironment is likely a major determinant of activity of cancer therapy, and thus acquisition of tumor stroma is also highly recommended.

Since biopsies are frequently small and not uniform, adequate sample management, quality control and/or prioritization is essential to accomplish robust biomarker research. Prolonged storage of samples under suboptimal conditions may compromise the quality of the sample analytes (e.g., proteins, phosphopeptides or nucleic acids) and interpretation of results. Definition of the optimal time periods, quality metrics, pre-analytical processing and conditions for sample/biomolecule preservation for biomarker studies is a major need in the field, but is beyond the scope of this article and has been reviewed elsewhere [134–137].

The procedures underlying the acquisition of quality tumor tissue were not detailed in any of the studies reviewed. Rapid on-site evaluation (ROSE) of tissue by a well-trained pathologist increases the yield of biopsies in the diagnostic setting [138], and also seems useful for biomarker studies.

Blood also represents a relevant platform for biomarker development. To date, it has mainly been used to characterize previously identified biomarkers (Table 1), with gefitinib being the first drug to obtain regulatory approval based on this strategy [139]. Blood was also the primary source in the characterization of *BRCA* germline mutations [104] and may be used to develop new biomarkers. For instance, upon validation, expression of the androgen receptor variant 7 (AR-V7) mRNA in circulating tumor cells (CTC) of prostate cancer patients might become a predictive biomarker of resistance to abiraterone and enzalutamide [140]. Sequencing of CTC may provide a comprehensive genomic characterization of tumors, avoiding invasive procedures [141]. Blood might also provide a more complete landscape of the disease at a systemic level, since it represents the genetic information from all tumor regions, rather than the limited image of the disease obtained by sampling a single tumor site, and may therefore contribute to solve the conundrum generated by tumor subclonal heterogeneity [142].

Blood extractions are minimally invasive and easy to standardize procedures, and the logistics required to manage them are already widespread. Therefore, blood-based biomarker research may vastly increase the availability of samples and consequently, our ability to identify biomarkers. Standardization of sample processing would foster the development of multi-institutional cooperative projects. Table 3 includes selected biomolecules that may be obtained from blood and other biological fluids.

### Timing of sample acquisition. Sequential samples

None of the studies reviewed controlled the interval between biopsy acquisition and treatment administration, confirming that this is a frequently overlooked variable, despite its potential relevance. This interval frequently depends on the natural history of the disease, and may range from few weeks or months (e.g., lung cancer) to several years (e.g., prostate cancer), thus impacting on the probability of variations in the tumor molecular profile over time and jeopardizing the interpretation of studies. Therefore, it seems reasonable to control this variable, perhaps with the exception of germline alterations [104]. Unfortunately, defining optimal intervals is an intricate task, as they may vary between different tumor types, patients, and even phases of the tumor evolution within one patient. Also, technical and ethical considerations limit access to tumor tissue at pre-specified intervals. Until more data becomes available, reporting the time elapsed between acquisition and initiation of therapy or the clinical event being evaluated; the disease setting of sample acquisition (i.e., localized vs. metastatic); and the location (primary tumor vs. metastasis) may help to interpret the data and to further define these concepts in the future.

Nevertheless, the development of molecular changes over time, rather than being an obstacle, represents a formidable opportunity to characterize their impact on the tumor phenotype. Indeed, the study of sequential biopsies obtained at baseline and at response, has allowed to characterize pharmacodynamic biomarkers [94].

**Table 3**  
Selected biomolecules that may be obtained from blood and other biological fluids in biomarker identification studies.

Biological sample	Type of sample	Analyte	Extraction			Storage		Observations <sup>f</sup>
			Collecting tube <sup>a</sup>	Volume (mL)	Centrifugation	Volume of aliquots (mL)	Long term storage <sup>b,c</sup>	
Blood	Serum	Metabolites Proteins Cytokines Exosomes Cell-free nucleic acids <sup>d</sup>	Clotting tubes	2.5–10	Wait 30 min to coagulation in vertical position Avoid long delays	0.3–0.5	–70 °C	Improper coagulation may influence downstream analysis.
	Plasma	Metabolites Proteins Cytokines Exosomes Cell-free nucleic acids <sup>d</sup>	K <sub>3</sub> EDTA, citrate or heparin tubes	2.5–10	Avoid long delays	0.3–0.5	–70 °C	Fibrin formation may influence downstream analysis
			K <sub>3</sub> EDTA or citrate tubes Cell-Free DNA <sup>TM</sup> BCT Cell-Free RNA <sup>TM</sup> BCT PAXgene tubes	5–10	Avoid long delays Follow specific protocols	0.5–1.5	–20 °C for weeks-months –70 °C	Stable for several days in special tubes at room temperature before isolation Extract nucleic acids before freezing Plasma DNA levels are > 3-fold lower than serum levels
	Whole blood	CTC	K <sub>3</sub> EDTA tubes CellSave <sup>TM</sup> preservative tubes	≥ 7.5	Follow specific protocols		Liquid nitrogen for viable cells	Follow protocols for viable cells if necessary
		Peripheral blood cells, Germline DNA <sup>e</sup>	K <sub>3</sub> EDTA or heparin tubes Cyto-Chex BCT CPT <sup>TM</sup> or PPT <sup>TM</sup>	≥ 2.5	Follow specific protocols (e.g., Ficoll)		Liquid nitrogen for viable cells	Follow protocols for viable cells if necessary
Urine	Random or 24 h	Metabolites Proteins Cytokines Exosomes Cell-free nucleic acids <sup>d</sup>	Specific lab recipient	Depends on sample	Avoid long delays Refrigerate until centrifugation	> 1	–70 °C	–
Other fluids (CSF, ascites, pleural fluid, saliva...)		Metabolites Proteins Cytokines Exosomes Cell-free nucleic acids CTC	Specific lab recipient	Depends on sample	Avoid long delays Refrigerate until centrifugation	0.3–1	–70 °C	Fibrin formation may influence downstream analysis

CSF: cerebrospinal fluid. CTC: circulating tumor cells.

<sup>a</sup> Some analytes may require special preservatives

<sup>b</sup> Avoid repeat freeze–thaw cycles

<sup>c</sup> Samples are viable for years

<sup>d</sup> Including miRNAs, lncRNAs, etc.)

<sup>e</sup> Although the effect of chemotherapy on germline DNA is not well characterized, it is recommended to obtain it at baseline, before treatment.

<sup>f</sup> Some results may be misrepresented by coexistence of concomitant diseases (e.g., hepatic or renal failure, etc).



**Table 4**

Selected cancer biomarkers related with acquired drug resistance identified or validated in sequential samples from initially sensitive patients or in synchronic lesions presenting paradoxical responses.

Reference	Tumor	Drug	n	Sampling strategy	Biomarker
Misale [142]	KRAS wild type colorectal cancer	Cetuximab/panitumumab	10	Sequential biopsies and circulating DNA	KRAS mutations
Diaz [146]	KRAS wild type colorectal cancer	Panitumumab	24	Sequential circulating DNA	KRAS mutations
Bettegowda [147]	KRAS wild type colorectal cancer	Cetuximab/panitumumab	24	Sequential circulating DNA	MAPK mutations
Pao [192]	EGFR mutated NSCLC	Gefitinib and Erlotinib	5	Sequential biopsies	EGFR T790 mutation
Choi [193]	NSCLC harboring ALK rearrangements	Crizotinib	1	Sequential biopsy	ALK mutations
Katayama [194]	NSCLC harboring ALK rearrangements	Crizotinib	18	Sequential biopsies	ALK mutations and amplification
Shaw [195]	NSCLC harboring ALK rearrangements	Lorlatinib	1	Sequential biopsies	ALK mutation <sup>a</sup>
Awad [196]	NSCLC harboring ROS1 rearrangement	Crizotinib	1	Sequential biopsy	ROS1 mutation
Emery [197]	Advanced melanoma	AZD6244 (MEK inhibitor)	5	Sequential biopsies	MEK1 mutation
Johannessen [198]	BRAF mutant melanoma	Vemurafenib	3	Sequential biopsies	MAPK pathway activation
Nazarian [199]	BRAF mutant melanoma	Vemurafenib	12	Sequential biopsies	PDGFR $\beta$ upregulation /NRAS mutation
Wagle [200]	BRAF mutant melanoma	Vemurafenib	1	Sequential biopsy	MEK1 mutation
Poulikakos [201]	BRAF mutant melanoma	Vemurafenib	19	Sequential biopsies	BRAF splicing variants
Shi [202]	BRAF mutant melanoma	Vemurafenib / dabrafenib	20	Sequential biopsies	(V600E)B-RAF amplification
Trunzer [203]	BRAF mutant melanoma	Vemurafenib	16	Sequential biopsies	MAPK signaling reactivation/ NRAS and MEK1 mutations
Van Allen [204]	BRAF mutant melanoma	Dabrafenib, vemurafenib	31	Sequential biopsies	MAPK pathway alterations, others
Ahronian [205]	BRAF mutant colorectal cancer	Dabrafenib and trametinib or panitumumab	3	Sequential biopsies	MAPK pathway alterations
Wagle [118]	Anaplastic thyroid cancer	Everolimus	1	Sequential biopsy	Somatic mTOR mutation
Cools [191]	Hypereosinophilic syndrome	Imatinib	1	Sequential biopsies	PDGFR $\alpha$ mutation
Debiec [206]	GIST	Imatinib	26	Sequential biopsies	PDGFR $\alpha$ and C-KIT mutations and amplification
Wardelmann [207]	GIST	Imatinib	32	Sequential biopsies	C-KIT mutations
Lim [208]	GIST	Imatinib	12	Sequential biopsies	PDGFR $\alpha$ and C-KIT mutations
Liegl [209]	GIST	Imatinib, sunitinib	14	Sequential samples	C-KIT mutations
Tamborini [210]	GIST	Imatinib	1	Synchronic biopsy	C-KIT mutations
Serrano [211]	GIST	Imatinib	1	Synchronic biopsy	KRAS and CKIT mutations
Zaretsky [212]	Melanoma	Pembrolizumab	4	Sequential biopsies	JAK1, JAK2 and B2Mtruncating mutations

GIST: gastrointestinal stromal tumors. NSCLC: non-small cell lung cancer.

<sup>a</sup> The patient developed previously a described ALK mutation that conferred resistance to crizotinib. She responded to lorlatinib and developed a second mutation that conferred resistance to lorlatinib, but resensitized the tumor to crizotinib.

In addition, the study of tumor specimens from patients that progress on treatment after initial responses has led to the identification of resistance biomarkers (Table 4), and to the development of new treatments directed to overcome resistance, as was the case with EGFR T790M mutations.[12] Interestingly, acquired resistance alterations were usually identified in the same driver genes or pathways that conferred sensitivity to treatment. Therefore, we hypothesize that whenever driver genes are unknown, new mutations that arise upon progression following a response, may constitute candidate resistance mutations that might be harbored in such driver genes or pathways, thus helping to identify them (reverse identification, Fig. 2). Sequencing such genes in baseline samples of responding patients may reveal the specific driver mutations.

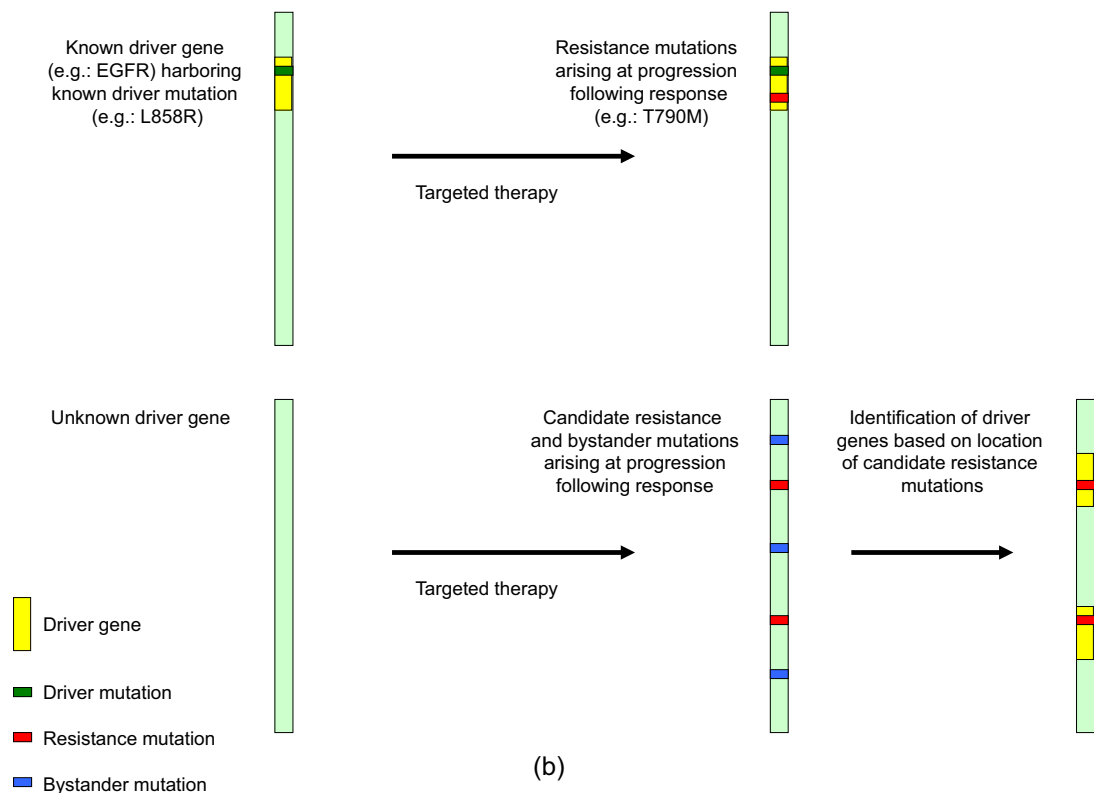
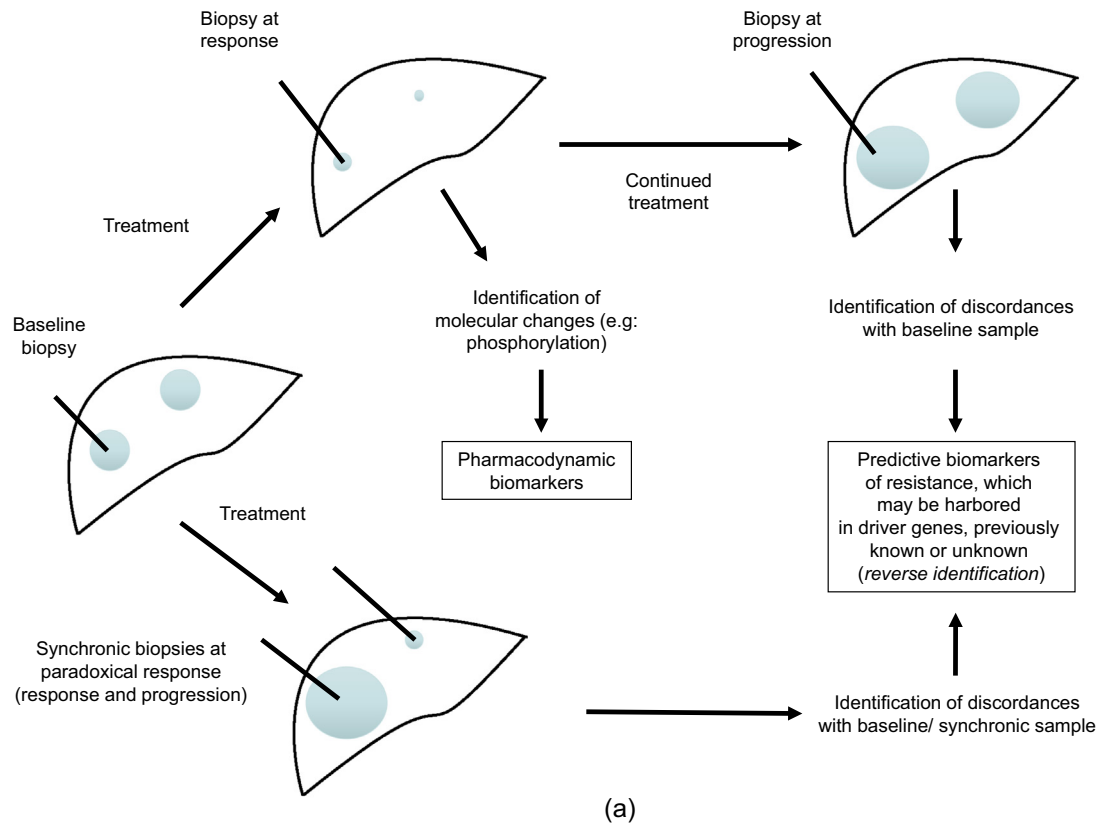
Synchronous biopsies of different lesions within a single patient that presents paradoxical responses to an anticancer drug (i.e., simultaneous response and progression) may also allow correlating phenotypic differences with the corresponding molecular profile and may help to improve our current understanding of tumor heterogeneity (Fig. 2, Table 4).

Sequential biopsies are not routinely performed in standard practice. Yet, some clinical situations allow sequential tissue

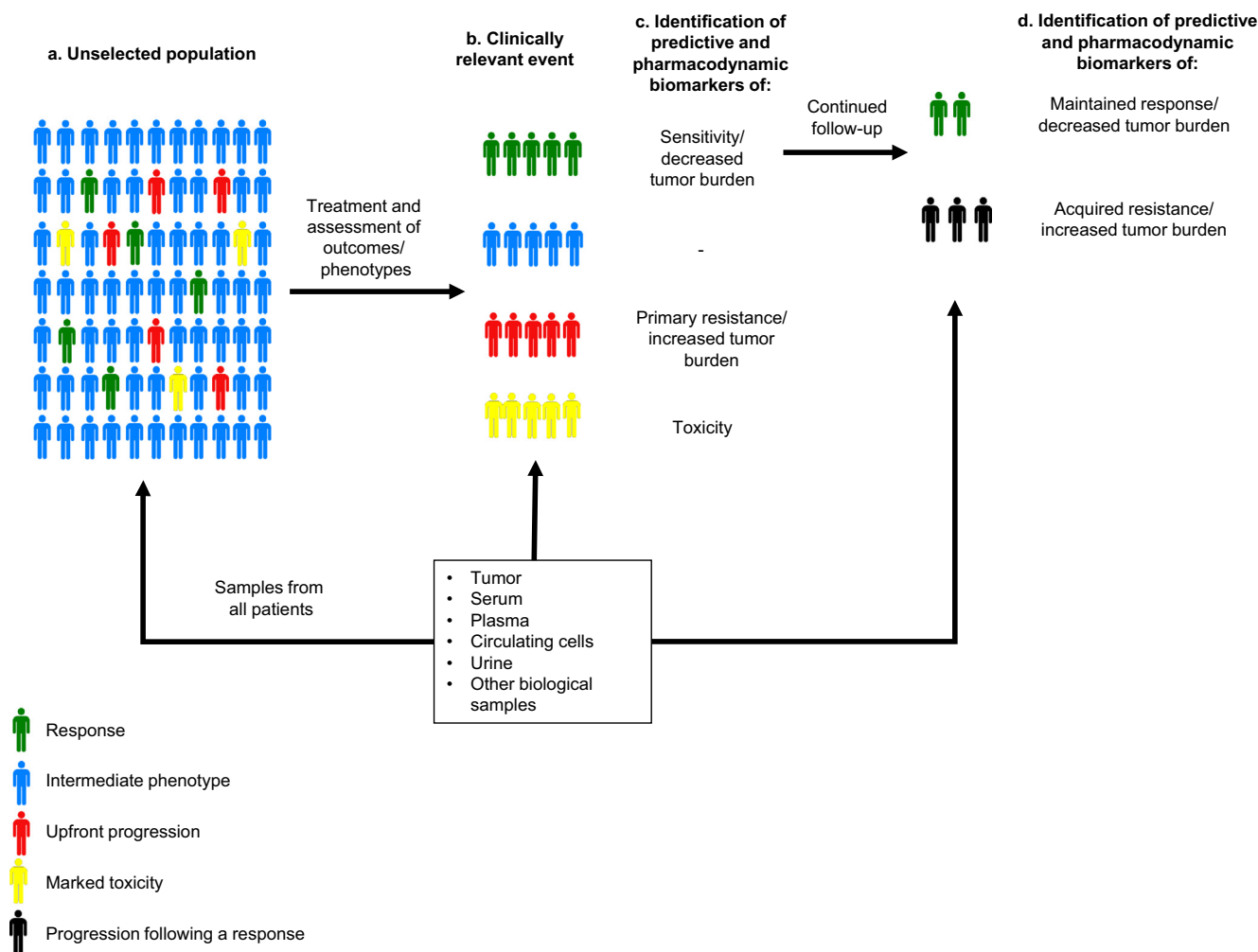
samples to be obtained in standard patient care, and thus represent excellent opportunities for biomarker research that should be pursued further. In responding patients, sequential tissue may be obtained from surgical resections following neoadjuvant therapy. Tumor may be obtained from patients presenting progression: at salvage surgery, following failure of induction therapy; to reassess the molecular profile to guide subsequent therapy; to resect tumor progressing at a single site; to assess pseudo-progression vs. true progression; or from autopsies, which have guided medical knowledge for centuries and may certainly have a role in the era of molecular biology. Finally, some tumor lesions, such as subcutaneous nodules, are readily accessible for sequential biopsies, thus entailing negligible risks and ethical concerns.

In the research setting, obtaining access to new drugs may compensate patients for the risks and inconveniences associated with investigational biopsies. Indeed, some trials with novel agents require for inclusion the acquisition of a tumor sample to be obtained following progression from a previous treatment (e.g., NCT01900652); or require to perform sequential biopsies during the study (e.g., NCT01358721).

Blood also constitutes an excellent platform to obtain sequential samples. Blood allows detection of predictive and



**Fig. 2.** Panel 2a: Design of studies exploring responses following progression or paradoxical responses. These studies require the acquisition and study of tumor biopsies at baseline and at progression, following a response to a given treatment; or from different tumor lesions presenting paradoxical responses (i.e.: response and progression). Comparison of baseline and responding lesions may identify pharmacodynamic biomarkers of efficacy. Differences in the molecular profile of baseline and progressing lesions may represent biomarkers of acquired resistance. Panel 2b: Since many of the described biomarkers of resistance occur on previously known driver genes or pathways (Table 4), it can be hypothesized that the identification of genetic alterations arising at resistance may help to identify the driver genes or pathways that harbor them, when these are unknown (*reverse identification*).



**Fig. 3.** Design of biomarker identification studies using sequential samples from patients treated with systemic therapies: a. Baseline samples are obtained from all patients treated with a particular drug. b. Sequential samples are obtained whenever a clinically relevant event (i.e., response, progression or marked toxicity) is observed. c. Baseline and sequential samples from patients presenting clinically relevant events may be interrogated to identify predictive or pharmacodynamic associated biomarkers. d. Sequential samples of patients presenting initial responses may be used to identify biomarkers associated with sustained response and/or decreased tumor burden; or with development of acquired resistance and/or increased tumor burden.

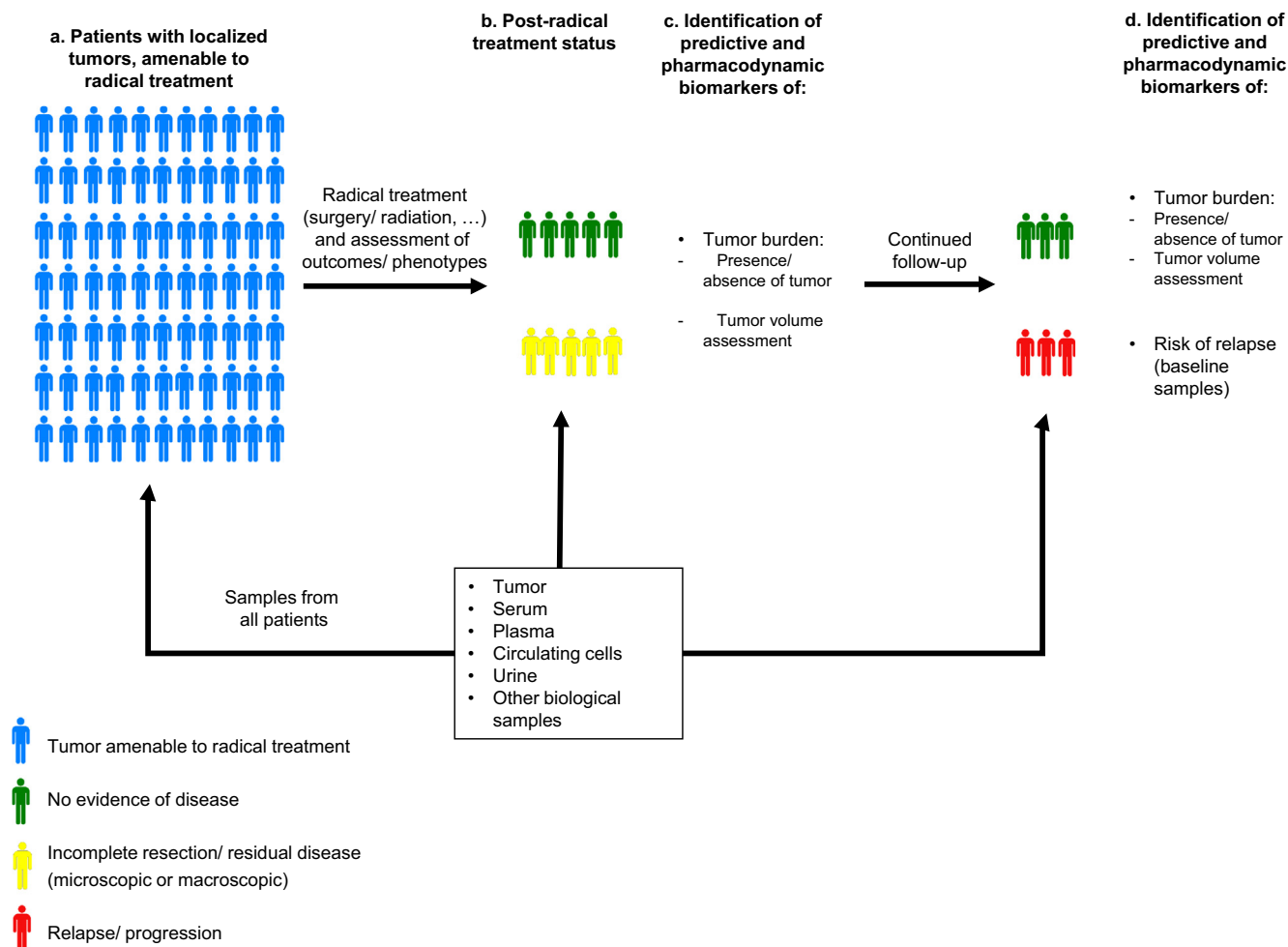
tumor-burden-related biomarkers, and monitoring of their levels over time, in order to evaluate tumor burden and response to treatment and to anticipate clinical progression [142–145] as well as development of resistance mutations [142,146,147], thus allowing early switch of therapy. Sequential blood samples should be obtained at clinically relevant moments, i.e., baseline, evaluation of response, or marked toxicity (Fig. 3). Samples obtained before and after radical treatment and at relapse are especially appropriate to identify prognostic, diagnostic and tumor-burden-related biomarkers (Fig. 4) [148]. Precise coordination in the collection of samples and clinical data is essential, and the role of well-trained and motivated research nurses, study coordinators and technicians in this task cannot be overemphasized.

#### Validation of biomarkers

Although prospective randomized trials are considered the gold standard to validate biomarkers, in fact they were not used to validate several of the biomarkers reviewed (Table 1). Randomized studies are time and resource consuming and raise ethical dilemmas, related to the denial of highly active treatments to control subjects [149]. Hence, it is necessary to critically evaluate if they are truly essential for biomarker validation.

Hormone receptors [20] and *KRAS* [78], *c-KIT* [37], and *BRCA* mutations [105] were validated based on overwhelming differences over historical controls and retrospective analyses of clinical trials. Even though randomized trials validated *ALK* translocations as a predictive biomarker for crizotinib [65,66], approval was granted before these trials ended, based on the striking results from the initial and confirmatory non-randomized studies [62,64]. Randomized trials validated *EGFR* [56–58] and *BRAF* mutations [95]. Nonetheless, the benefit for *EGFR* mutated patients treated with TKI was so remarkable that the biomarker was adopted before the results of phase III trials became available [150,151]. Moreover, novel *EGFR* mutations, not assessed in randomized trials have been incorporated into clinical practice as predictive biomarkers for *EGFR* TKI. As for *BRAF* inhibitors, randomization involved ethical dilemmas due to the obvious superiority of the experimental treatment [149]. Finally, randomized trials were pivotal in the validation of *HER2* overexpression in breast [27] and gastric cancer [29]. However, the activity of trastuzumab in patients expressing *HER2* is lower than for the other biomarkers, and probably these subtler differences in efficacy make the randomized validation essential.

*Basket trials*, which enroll patients with different tumor types according to the expression of molecular alterations, constitute a



**Fig. 4.** Design of biomarker identification studies using sequential samples from patients with localized tumors, amenable to radical therapy (i.e., surgery or radiation): a. Baseline samples are obtained from all patients before radical therapy. b. Sequential samples are obtained following radical therapy. c. Baseline and sequential samples from patients treated with radical therapy may be interrogated to identify predictive or pharmacodynamic biomarkers of tumor burden and risk of relapse (baseline samples). d. Baseline and sequential samples from continued follow-up of these patients may be interrogated to identify biomarkers of tumor burden and risk of relapse (baseline samples).

flexible and interesting design to validate new candidate predictive biomarkers; or to validate in new tumor types biomarkers that are already well-established in others. The NCI Molecular Analysis for Therapy Choice (MATCH) trial, which will screen 4,000 different variants across 143 genes in over 5,000 patients and assign patients carrying specific alterations to 24 treatment arms, constitutes a groundbreaking initiative in this field [152]. *Umbrella studies*, which assess several genetic alterations on a given tumor type and assign treatments accordingly, also constitute a relevant tool to further explore and validate biomarkers.

#### *Related to the molecular aspects of the biomarkers*

##### Molecular nature of the biomarker

Biomarkers represent molecular features of tumors that activate or repress biological pathways that drive neoplastic growth, thus rendering the tumor sensitive or resistant to drugs affecting that pathway. Theoretically, such features might be found at different levels of the cellular machinery as exemplified by HER2 overexpression, which is detectable at the gene and/or at the protein levels. To date all cancer predictive biomarkers consist of alterations at the genetic or protein expression levels (Table 1), and even though other types of biomarkers have been reported, none has been translated into clinical practice (Table 5). Consequently,

it seems logical to prioritize gene and protein evaluation in biomarker identification studies.

These levels also apply to cancer immunotherapy biomarkers, with PD-L1 expression [153] and presence and clonality of tumor neoantigens [154–156] being the most relevant examples for immunomodulatory antibodies. Yet, the characteristics of the stroma and the immune infiltrate [157–159] and the functionality of the immune system [160] must also be considered in the identification of robust biomarkers in this field. The complexity of the immune system may require the use of quantitative or semiquantitative scores assessing different variables [161] that should be correlated with benefit of single agent or combination immunotherapy [162].

##### Preclinical evidence

Preclinical evidence is available for all the biomarkers reviewed and, in most instances, preceded clinical discovery (Table 1). Nevertheless *EGFR* [52,53] and *KRAS* mutations [78] were first described in patients and validated subsequently in preclinical models, although, even in these cases, preclinical knowledge of the signaling pathways guided the clinical studies.

Even though modern high-throughput techniques assess countless molecular alterations, just a limited number of these seem critical for tumor development (i.e., “driver” tumor alterations),

**Table 5**

Molecular nature of biological alterations for selected cancer predictive biomarkers.

Level	Molecular alteration	Example	Diagnostic test	Tumor type	Treatment	Validated	Approved for clinical use
Genetic	Gene mutations and deletions	<i>EGFR</i> mutations [52,53]	PCR, sequencing	NSCLC	<i>EGFR</i> TKI	Yes	Yes
		<i>c-KIT</i> mutations [37]	PCR, sequencing	GIST	Imatinib, sunitinib	Yes	Yes
		<i>KRAS/ NRAS</i> mutations [78,85]	PCR, sequencing	Colorectal cancer	Cetuximab, panitumumab	Yes	Yes
	Gene amplification	<i>HER2</i> overexpression [26]	IHC, FISH	Breast cancer Gastric cancer	<i>HER2</i> targeted therapy	Yes	Yes
	Translocations	<i>ALK</i> translocation [61]	FISH	NSCLC	<i>ALK</i> inhibitors	Yes	Yes
	Polymorphisms	<i>VEGFR3</i> SNPs [213]	PCR	RCC	Sunitinib	No	No
Epigenetic	Messenger RNA	<i>ARV7</i> [140]	PCR	Prostate Cancer	Abiraterone, enzalutamide	No	No
	miRNA	miR-942 [214]	Micro RNA array	RCC	Sunitinib	No	No
	DNA methylation	MGMT methylation [215]	PCR	Glioblastoma	Alkylating agents	No	No
Protein	Protein overexpression	<i>HER2</i> [26]	IHC	Breast cancer Gastric cancer	<i>HER2</i> targeted therapy	Yes	Yes
		Hormone receptors [20]	IHC	Breast cancer	Hormonal therapy	Yes	Yes
Phenotype	Cellular subpopulations	CD8+ tumor infiltrating lymphocytes [157]	IHC	Melanoma	PD-1/ PD-L1 axis blocking therapy	No	No

AR-V7: androgen receptor variant 7. EGFR: epidermal growth factor receptor. FISH: fluorescence in-situ hybridization. IHC: immunohistochemistry. MGMT: O6-methyl-guanine-DNA methyltransferase PCR: polymerase chain reaction. RCC: renal cell carcinoma. SNPs: single nucleotide polymorphisms. TKI: tyrosine kinase inhibitors.

**Table 6**

Sample requirements for selected molecular and pathological techniques in the assessment and discovery of cancer predictive biomarkers.

Technique	Application/s	Sample requirement <sup>a</sup>	Analytical Sensitivity	Observations
PCR-based: conventional PCR, pyrosequencing, Sanger sequencing, RFLP, RT-PCR, ASO, etc	<i>EGFR</i> , <i>KRAS/NRAS</i> , <i>BRAF</i> , <i>c-KIT</i> mutation	>5–10 ng (approx. 1000 cells)	From 3% for pyrosequencing to 15% for Sanger sequencing	May depend on the sensitivity and specificity of the technique
Methylation specific: pyrosequencing/MSP-PCR	MGMT methylation	1 µg	3–5% of methylated DNA	
NGS: gene panels	Hot spots or complete coding sequence of target genes: assessment and discovery	20 ng to 1 µg	Variable	Highly dependent on the type of library and equipment.
NGS, WES	Exome analysis: discovery	2–3 µg	Variable	Highly dependent on the type of library and equipment. Not for FFPE.
GWAS	Discovery and identification of SNPs or loci related to the phenotype under study	1 µg of DNA from peripheral blood	Variable	
FISH	<i>ALK</i> and <i>ROS1</i> rearrangements	FFPE sections with at least 50–100 cancer cells, cytology smears	15% of rearranged cells	FISH is not validated for cytology smears, when used, negativity in the smear does not exclude the possibility that the tumor contains translocated genes
IHC	<i>HER2</i> overexpression, hormonal receptors, <i>ALK</i> IHC Test (Ventana), CD8+ tumor infiltrating lymphocytes	Cytology smear, FFPE sections	15% of positive cells	

ASO: Allele specific oligonucleotide; FFPE: formalin-fixed paraffin-embedded tissue; GWAS: Genome-wide association study. FISH: Fluorescence in situ hybridization. IHC: Immunohistochemistry. MSP-PCR: Methylation specific polymerase chain reaction; NGS: Next generation sequencing; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; WES: Whole exome sequencing.

<sup>a</sup> In all cases the DNA optical density (OD) 260/280 must be between 1.8 and 2.0.

as opposed to others that seem to be bystanders in the process of carcinogenesis (“passenger” alterations) [163]. Also, while sometimes the biomarkers consist of a unique molecular alteration, identical in all patients (e.g., *BRAF* V600E mutation) [94], in other instances they are represented by diverse molecular alterations that induce comparable phenotypes (e.g., the different *EGFR* sensitizing mutations) [52,53]. Moreover, due to tumor heterogeneity and clonal evolution, different tumor lesions may present different molecular alterations. This biological diversity poses a challenge for biomarker discovery using unsupervised methods and renders preclinical validation essential for biomarker identification. Com-

putational tools to estimate pathogenicity of genetic alterations based on its location, frequency or predicted structural impact may help to select candidate variants, but it is unlikely that they may substitute functional validation in a preclinical model. Preclinical evidence is also fundamental to identify mechanisms of acquired resistance to targeted drugs and to guide strategies to overcome them [164–167].

#### Conventional vs high-throughput techniques

Identification of biomarkers is inherently related to the technological capacities to analyze samples/analytes and correlate the



**Table 7**  
Summary of conclusions.

1. Even though prospective studies are the gold standard for medical research, retrospective designs also represent a useful tool to identify predictive cancer biomarkers and should be considered for this purpose.
2. Single-agent studies should be preferred over combination studies to identify biomarkers.
3. Advanced disease seems the more appropriate setting to perform biomarker identification studies, due to methodological and logistical advantages.
4. Response rate has been the most widely used endpoint for studies to identify predictive biomarkers, because of its direct correlation with drug activity. Nevertheless, other endpoints may also be used.
5. Extreme phenotype selection is a useful strategy for biomarker identification studies, because it enriches the expression of biomarkers, allows lower sample sizes to be used and aids in the interpretation of the large amounts of data generated by high-throughput techniques.
6. The development of reliable statistical methods to calculate the optimal sample size for biomarker identification studies is an unmet need. Until such methods are available, empirical determination of sample size, based on currently available successful experiences, may be an adequate approach.
7. Tumor tissue remains the preferred sample for biomarker identification studies. Acquisition of adequate samples of paraffin-embedded and frozen tissue is paramount to develop solid biomarker research programs. Strategies to increase the yield of biopsies, such as Rapid On-Site Evaluation (ROSE) of samples by a pathologist should be encouraged.
8. Blood is an excellent platform for biomarker research. To date, blood has mainly been used to detect already known biomarkers, but it also may be used for primary identification of biomarkers.
9. Timing of sample acquisition is a relevant variable that should be controlled in biomarker identification studies, although the optimal and maximum intervals between sample acquisition and the clinical event being evaluated remain to be defined. Reporting these intervals in biomarker identification studies should help to define them in the future.
10. Studies with sequential biological samples, obtained at clinically relevant moments are an excellent platform to identify predictive biomarkers. Biopsies obtained at progression have revealed resistance mechanisms in driver genes, and may be useful to discover new driver genes. Synchronous biopsies of tumor lesions presenting paradoxical responses are also useful for this purpose. Blood is especially appropriate for biomarker studies with sequential samples.
11. Although randomized trials remain the gold standard to validate candidate biomarkers, non-randomized approaches have also been successful. The confirmation of large differences in efficacy in comparison with historical controls in one or more prospective –or even retrospective– studies has been a widely accepted strategy for this purpose.
12. All validated predictive biomarkers available have been identified at the genetic and/ or protein expression levels. Consequently, it seems logical to prioritize such levels.
13. Preclinical evidence is essential to confirm the reliability of candidate predictive biomarkers. It can be obtained either upfront, to guide clinical studies; or subsequently, to validate the results of clinical studies.
14. Regulatory authorities and pharmaceutical industry should discuss and implement an adequate regulatory framework to further support drug development based on biomarkers.
15. Ethical regulations should guarantee the rights of the individuals without compromising the development of translational research. The development of broad research projects with one-time informed consents seems a useful tool for this purpose.

results with clinical outcomes. Genomic, transcriptomic, epigenetic and protein profiling can determine up to thousands of markers simultaneously, with continuously decreasing time frames, costs and sample requirements, and will likely dominate the field in the coming years. However, all the predictive biomarkers reviewed were identified using relatively simple, low throughput methods, such as immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), quantitative PCR, capillary electrophoresis and hotspot/targeted sequencing. Even though some high-throughput methods, such as gene arrays help to select patients for adjuvant therapy, for example in breast cancer [168–171], these cannot be considered predictive biomarkers for targeted agents. Table 6 reviews some of the most widely used conventional and high-throughput techniques commonly used for biomarker research.

Theoretically, high-throughput technologies using unbiased/unsupervised discovery approaches should increase the ability to find biomarkers, perhaps allowing identification of initially unexpected candidates; selection of the optimal biomarker, whenever several candidates are identified; or the development of composite biomarkers. Yet, high-throughput techniques provide an extremely large number of variables that are difficult to interpret reliably. In addition, performing multiple comparisons leads to many false significant associations. Several methods to adjust for multiple testing are available, with the preferred approach being to control the false discovery rate (FDR), which represents the probability that any particular significant finding represents a false positive result [172]. Biomarker identification approaches are normally based on pattern matching algorithms [173,174]. Classification is the process of finding a model that distinguishes data classes based on the analysis of a training population (subjects whose class label is known). Once the model is established, it is applied to one or more independent validation sets to challenge its capacity to predict the class in a population whose class label is unknown. This controls for statistical overfitting and any particular population/

selection bias. Evaluation of biomarker performance in independent data sets is cumbersome, and thus statistical approaches based on cross-validation or bootstrapping are commonly used [175]. One of the most salient issues for biomarker discovery is that datasets are inevitably biased by subject selection. As mentioned above, sampling individuals (cases and controls) from the extremes of a quantitative distribution (observable or inferred from a statistical model) may increase power [176,177].

Therefore, while high-throughput technologies may generate a great number of candidate biomarkers with potential clinical value, to date their use remains mainly exploratory and directed towards the screening of candidate biomarkers. Standardization of high-throughput sequencing-based methods across laboratories and incorporation of novel statistical approaches will be required to develop more efficient biomarker discovery programs.

### Regulatory and ethical aspects

Despite the efforts of regulatory agencies and pharmaceutical companies to define drug development based on biomarkers [178], few drugs as yet follow that path, as compared with those developed based on conventional phase III studies. This situation probably indicates that the regulatory authorities and industry need to keep collaborating to define a regulatory environment that further supports biomarker-based drug development.

As for ethical aspects, legislators and ethical review boards must guarantee the rights of the individuals that donate biologic samples according to the highest standards, while they must also acknowledge that the availability of human samples is a key limiting factor in cancer research; and that most patients are willing to collaborate in this purpose and trust researchers to act ethically [179].

Research projects with broad and comprehensive one-time informed consents that contain all the information required by

legal authorities and ethical review boards seem an adequate strategy to protect the patient's rights without compromising the development of translational research. Such projects should allow investigators to interrogate the samples with a wide variety of molecular techniques, including high-throughput strategies and the development of *in vitro* and *in vivo* models. They should provide a contact point, where patients may exert forthcoming rights in the future: obtain additional information, withdraw consent, etc.; and they should be adequately monitored by ethical review boards.

Instead, stringent interpretations of legislations that demand to define the specific biomarker that is being pursued –which is unknown, by definition–, to provide technical details (i.e., laboratory procedures, location of the research laboratories, etc.), or to contact patients again whenever any ancillary condition is modified, severely restrain investigators from optimizing the yield of the samples and are not generally demanded by patients.

The implementation of broad research projects should decrease the administrative and financial burden dedicated to project management; and should increase the number of samples available for translational research. The establishment of homogeneous policies at an international level would also simplify the development of translational projects across country borders, as is the case with clinical trials (i.e., Good Clinical Practice guidelines). This would be especially relevant for the identification and study of patients presenting very infrequent extreme phenotypes.

## Conclusions

The identification of predictive biomarkers is one of the greatest challenges of cancer research. While major advances have been achieved in this field, solid methodological designs must be developed to maximize our potential to identify new biomarkers.

Despite the intrinsic complexity of this field, several biomarkers are already available for clinical use, and it is rewarding to confirm that the time required to identify reliable predictive biomarkers has decreased dramatically in recent years (Table 1). This experience should guide the design of studies to identify predictive biomarkers (Table 7). Since the technology is already available, this effort will certainly accelerate progress in this pivotal field and will foster the development of personalized medicine, which in the end will require personalized research.

## Conflict of interest

The authors declare no conflicts of interest with regard to the content of this manuscript.

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## References

- [1] Sawyers CL. The cancer biomarker problem. *Nature* 2008;452:548–52. [nature06913 \[pii\]10.1038/nature06913](#).
- [2] Jiang W, Freidlin B, Simon R. Biomarker-adaptive threshold design: a procedure for evaluating treatment with possible biomarker-defined subset effect. *J Natl Cancer Inst* 2007;99:1036–43. [10.1093/jnci/djm022](#).
- [3] Mandrekar SJ, Sargent DJ. Clinical trial designs for predictive biomarker validation: one size does not fit all. *J Biopharm Stat* 2009;19:530–42. [Doi: 910605768 \[pii\] 10.1080/10543400902802458](#).
- [4] Mandrekar SJ, Sargent DJ. Clinical trial designs for predictive biomarker validation: theoretical considerations and practical challenges. *J Clin Oncol* 2009;27:4027–34. [Doi: JCO.2009.22.3701 \[pii\]10.1200/JCO.2009.22.3701](#).
- [5] Buyse M, Sargent DJ, Grothey A, Matheson A, de Gramont A. Biomarkers and surrogate end points—the challenge of statistical validation. *Nat Rev Clin Oncol* 2010;7:309–17. [doi: nrcinonc.2010.43 \[pii\]10.1038/nrcinonc.2010.43](#).
- [6] Mandrekar SJ, Sargent DJ. Predictive biomarker validation in practice: lessons from real trials. *Clin Trials* 2010;7:567–73. [doi: 1740774510368574 \[pii\] 10.1177/1740774510368574](#).
- [7] Buyse M, Michiels S, Sargent DJ, Grothey A, Matheson A, de Gramont A. Integrating biomarkers in clinical trials. *Expert Rev Mol Diagn* 2011;11:171–82. [doi: 10.1586/erm.10.120](#).
- [8] Issaq HJ, Waybright TJ, Veenstra TD. Cancer biomarker discovery: opportunities and pitfalls in analytical methods. *Electrophoresis* 2011;32:967–75. [10.1002/elps.201000588](#).
- [9] Marchiò C, Dowsett M, Reis-Filho JS, Marchio C. Revisiting the technical validation of tumour biomarker assays: how to open a Pandora's box. *BMC Med* 2011;9:41. [doi: 1741-7015-9-41 \[pii\]10.1186/1741-7015-9-41](#).
- [10] Mandrekar SJ, Sargent DJ. All-comers versus enrichment design strategy in phase II trials. *J Thorac Oncol* 2011;6:658–60. [doi: 10.1097/JTO.0b013e31820e17cb](#).
- [11] Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 2012;367:1694–703. [doi: 10.1056/NEJMoa1210093](#).
- [12] Jänne PA, Yang JC-H, Kim D-W, Planchard D, Ohe Y, Ramalingam SS, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *N Engl J Med* 2015;372:1689–99. [doi: 10.1056/NEJMoa1411817](#).
- [13] Shaw AT, Kim D-W, Mehra R, Tan DSW, Felip E, Chow LQM, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. *N Engl J Med* 2014;370:1189–97. [doi: 10.1056/NEJMoa1311107](#).
- [14] Chu E, DeVita VT. Physicians' Cancer Chemotherapy Drug Manual 2014:2014.
- [15] A to Z List of Cancer Drugs – National Cancer Institute n.d. <http://www.cancer.gov/cancertopics/druginfo/alphalist> (accessed December 23, 2014).
- [16] Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestion for a new method of treatment, with illustrative cases. *Lancet* 1896;148:162–5.
- [17] Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. *Clin Cancer Res* 2003;9:1980–9.
- [18] Jensen EV, Jacobson H. Basic guides to the mechanism of estrogen action. *Recent Prog Horm Res* 1962;18:318–414.
- [19] Toft D, Shyamala G, Gorski J. A receptor molecule for estrogens: studies using a cell-free system. *Proc Natl Acad Sci USA* 1967;57:1740–3.
- [20] Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. *Natl Cancer Inst Monogr* 1971;34:55–70.
- [21] Shih C, Padhy LC, Murray M, Weinberg RA. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* 1981;290:261–4.
- [22] King CR, Kraus MH, Aaronson SA. Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science* 1985;229:974–6.
- [23] Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. *Cell* 1989;57:931–6. [doi: 0092-8674\(89\)90331-0](#).
- [24] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;235:177–82.
- [25] Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. P185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol* 1989;9:1165–72.
- [26] Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol* 1996;14:737–44.
- [27] Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;344:783–92. [doi: 10.1056/NEJM200103153441101](#).
- [28] Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 2005;353:1659–72. [doi: 353/16/1659 \[pii\]10.1056/NEJMoa052306](#).
- [29] Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010;376:687–97. [doi: S0140-6736\(10\)61121-X \[pii\]10.1016/S0140-6736\(10\)61121-X](#).
- [30] Baselga J, Cortés J, Kim S-B, Im S-A, Hegg R, Im Y-H, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med* 2012;366:109–19. [doi: 10.1056/NEJMoa113216](#).
- [31] Verma S, Miles D, Gianni L, Krop IE, Welslau M, Baselga J, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N Engl J Med* 2012;367:1783–91. [doi: 10.1056/NEJMoa1209124](#).
- [32] Besmer P, Murphy JE, George PC, Qiu FH, Bergold PJ, Lederman L, et al. A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. *Nature* 1986;320:415–21. [doi: 10.1038/320415a0](#).

- [33] Nagata H, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci USA* 1995;92:10560–4.
- [34] Zureikat A, Heller M, Zeh H. Cancers of the small intestine. In: DeVita, Hellman, Rosenberg's Cancer Princ. Pract. Oncol.. Philadelphia: Wolters Kluwer Health-Lippincott Williams & Wilkins; 2015. p. 1056.
- [35] Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. *Blood* 2000;96:925–32.
- [36] Tuveson DA, Willis NA, Jacks T, Griffin JD, Singer S, Fletcher CD, et al. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. *Oncogene* 2001;20:5054–8. doi: 10.1038/sj.onc.1204704.
- [37] Joensuu H, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, et al. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N Engl J Med* 2001;344:1052–6. doi: 10.1056/NEJM200104053441404.
- [38] Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002;347:472–80. doi: 10.1056/NEJM0204613477/472 [pii].
- [39] Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen C-J, Joseph N, et al. PDGFRα activating mutations in gastrointestinal stromal tumors. *Science* 2003;299:708–10. doi: 10.1126/science.1079666.
- [40] Rubin BP, Schuetz SM, Eary JF, Norwood TH, Mirza S, Conrad EU, et al. Molecular targeting of platelet-derived growth factor B by imatinib mesylate in a patient with metastatic dermatofibrosarcoma protuberans. *J Clin Oncol* 2002;20:3586–91.
- [41] Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* 1962;237:1555–62.
- [42] Cohen S, Carpenter G, King Jr L. Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *J Biol Chem* 1980;255:4834–42.
- [43] Sporn MB, Todaro GJ. Autocrine secretion and malignant transformation of cells. *N Engl J Med* 1980;303:878–80. doi: 10.1056/NEJM198010093031511.
- [44] Moyer JD, Barbacci EG, Iwata KK, Arnold L, Boman B, Cunningham A, et al. Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 1997;57:4838–48.
- [45] Ciardiello F, Caputo R, Bianco R, Damiano V, Pomato G, De Placido S, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000;6:2053–63.
- [46] Fukuoaka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol* 2003;21:2237–46. doi:10.1200/JCO.2003.10.038 [pii].
- [47] Perez-Soler R, Chachoua A, Hammond LA, Rowinsky EK, Huberman M, Karp D, et al. Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. *J Clin Oncol* 2004;22:3238–47. doi:10.1200/JCO.2004.11.05722/16/3238 [pii].
- [48] Herbst RS, Prager D, Hermann R, Fehrenbacher L, Johnson BE, Sandler A, et al. TRIBUTE: a phase III trial of erlotinib hydrochloride (OSI-774) combined with carboplatin and paclitaxel chemotherapy in advanced non-small-cell lung cancer. *J Clin Oncol* 2005;23:5892–9. doi: 10.1200/JCO.2005.02.840.
- [49] Gatzemeier U, Pluzanska A, Szczesna A, Kaukel E, Roubec J, De Rosa F, et al. Phase III study of erlotinib in combination with cisplatin and gemcitabine in advanced non-small-cell lung cancer: the Tarceva Lung Cancer Investigation Trial. *J Clin Oncol* 2007;25:1545–52.
- [50] Giaccone G, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial-INTACT 1. *J Clin Oncol* 2004;22:777–84.
- [51] Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial-INTACT 2. *J Clin Oncol* 2004;22:785–94.
- [52] Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500. <http://dx.doi.org/10.1126/science.1099314>.
- [53] Lynch TJ, Bell DW, Sordella R, Gurubhagavata S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–39.
- [54] Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004;101:13306–11.
- [55] Han SW, Kim TY, Hwang PG, Jeong S, Kim J, Choi IS, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 2005;23:2493–501. doi: JCO.2005.01.388 [pii]10.1200/JCO.2005.01.388.
- [56] Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–8. doi: 362/25/2380 [pii]10.1056/NEJM0909530.
- [57] Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;11:121–8. doi: S1470-2045(09)70364-X [pii] 10.1016/S1470-2045(09)70364-X.
- [58] Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 2011;12:735–42. doi: S1470-2045(11)70184-X [pii]10.1016/S1470-2045(11)70184-X.
- [59] Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007;448:561–6. doi: nature05945 [pii] 10.1038/nature05945.
- [60] McDermott U, Iafrate AJ, Gray NS, Shioda T, Classon M, Maheswaran S, et al. Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. *Cancer Res* 2008;68:3389–95. doi: 68/9/3389 [pii]10.1158/0008-5472.CAN-07-6186.
- [61] Kwak EL, Camidge DR, Clark J, Shapiro GI, Maki RG, Ratain MJ, et al. Clinical activity observed in a phase I dose escalation trial of an oral c-met and ALK inhibitor, PF-02341066. *ASCO Meet Abstr* 2009;27:3509.
- [62] Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–703. doi: 10.1056/NEJM01006448.
- [63] Riely GJ, Kim DW, Crinó L, Janne PA, Blackhall FH, Camidge DR, et al. Phase 2 Data for Crizotinib (PF-02341066) in ALK-Positive Advanced Non-Small Cell Lung Cancer (NSCLC): PROFILE 1005. 14th World Conf. Lung Cancer, Amsterdam; 2011.
- [64] Shaw AT, Yeap BY, Solomon BJ, Riely GJ, Gainor J, Engelman JA, et al. Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: a retrospective analysis. *Lancet Oncol* 2011;12:1004–12. doi: S1470-2045(11)70232-7 [pii]10.1016/S1470-2045(11)70232-7.
- [65] Shaw AT, Kim D-W, Nakagawa K, Seto T, Crinó L, Ahn M-J, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med* 2013;368:2385–94. doi: 10.1056/NEJM01214886.
- [66] Solomon BJ, Mok T, Kim D-W, Wu Y-L, Nakagawa K, Mekhail T, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med* 2014;371:2167–77. doi: 10.1056/NEJM01408440.
- [67] Butrynski JE, D'Adamo DR, Hornick JL, Dal Cin P, Antonescu CR, Jhanwar SC, et al. Crizotinib in ALK-rearranged inflammatory myofibroblastic tumor. *N Engl J Med* 2010;363:1727–33. doi: 10.1056/NEJM01007056.
- [68] Gambacorti-Passerini C, Messa C, Pogliani EM. Crizotinib in anaplastic large-cell lymphoma. *N Engl J Med* 2011;364:775–6. doi: 10.1056/NEJMc1013224.
- [69] Pogliani EM, Dilda I, Villa F, Farina F, Giudici G, Guerra L, et al. High response rate to crizotinib in advanced, chemoresistant ALK+ lymphoma patients. *ASCO Meet Abstr* 2011;29:e18507.
- [70] Bergethon K, Shaw AT, Ou S-HI, Katayama R, Lovly CM, McDonald NT, et al. ROS1 rearrangements define a unique molecular class of lung cancers. *J Clin Oncol* 2012;30:863–70. doi: 10.1200/JCO.2011.35.6345.
- [71] Shaw AT, Ou S-HI, Bang Y-J, Camidge DR, Solomon BJ, Salgia R, et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. *N Engl J Med* 2014;371:1963–71. <http://dx.doi.org/10.1056/NEJM01406766>.
- [72] Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH. Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* 1983;1:511–29.
- [73] Fan Z, Lu Y, Wu X, Mendelsohn J. Antibody-induced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells. *J Biol Chem* 1994;269:27595–602.
- [74] Goldstein NI, Prewett M, Zuklys K, Rockwell P, Mendelsohn J. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin Cancer Res* 1995;1:1311–8.
- [75] Saltz LB, Meropol NJ, Loehrer Sr PJ, Needle MN, Kopit J, Mayer RJ. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 2004;22:1201–8. <http://dx.doi.org/10.1200/JCO.2004.10.1821>.
- [76] Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004;351:337–45. <http://dx.doi.org/10.1056/NEJM033025351/4/337>.
- [77] Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 2007;25:1658–64. <http://dx.doi.org/10.1200/JCO.2006.08.1620>.
- [78] Lievre A, Bachelot JB, Le Corre D, Boige V, Landi B, Emile JF, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 2006;66:3992–5. doi: 66/8/3992 [pii]10.1158/0008-5472.CAN-06-0191.
- [79] Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, et al. Oncogenic activation of the RAS/RAF signaling pathway



- impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res* 2007;67:2643–8. <http://dx.doi.org/10.1158/0008-5472.CAN-06-4158>.
- [80] De Roock W, Piessevaux H, De Schutter J, Janssens M, De Hertogh G, Personeni N, et al. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann Oncol* 2008;19:508–15. doi: [mdm496](https://doi.org/10.1093/annonc/mdm496) [pii]10.1093/annonc/mdm496.
  - [81] Lièvre A, Bachet J-BB, Boige V, Cayre A, Le Corre D, Buc E, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 2008;26:374–9. doi: [10.1200/JCO.2007.12.5906](https://doi.org/10.1200/JCO.2007.12.5906).
  - [82] Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;26:1626–34. doi: [10.1200/JCO.2007.14.7116](https://doi.org/10.1200/JCO.2007.14.7116) [pii]10.1200/JCO.2007.14.7116.
  - [83] Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;359:1757–65. doi: [359/17/1757](https://doi.org/10.1056/NEJMoa0804385) [pii]10.1056/NEJMoa0804385.
  - [84] De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilaz G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 2010;11:753–62. [http://dx.doi.org/10.1016/S1470-2045\(10\)70130-3](http://dx.doi.org/10.1016/S1470-2045(10)70130-3).
  - [85] Douillard J-Y, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med* 2013;369:1023–34. <http://dx.doi.org/10.1056/NEJMoa1305275>.
  - [86] Rapp UR, Goldsborough MD, Mark GE, Bonner TI, Groffen J, Reynolds Jr FH, et al. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. *Proc Natl Acad Sci USA* 1983;80:4218–22.
  - [87] Ikawa S, Fukui M, Ueyama Y, Tamaoki N, Yamamoto T, Toyoshima K. B-raf, a new member of the raf family, is activated by DNA rearrangement. *Mol Cell Biol* 1988;8:2651–4.
  - [88] Smalley KS. A pivotal role for ERK in the oncogenic behaviour of malignant melanoma? *Int J Cancer* 2003;104:527–32. <http://dx.doi.org/10.1002/ijc.10978>.
  - [89] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949–54. <http://dx.doi.org/10.1038/nature00766>.
  - [90] Ribas A, Flaherty KT. BRAF targeted therapy changes the treatment paradigm in melanoma. *Nat Rev Clin Oncol* 2011;8:426–33. [nrclinonc.2011.69](https://doi.org/10.1038/nrclinonc.2011.69) [pii]10.1038/nrclinonc.2011.69.
  - [91] Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 2004;116:855–67. [S0092867404002156](https://doi.org/10.1016/j.cell.2004.04.021).
  - [92] Dhomen N, Reis-Filho JS, da Rocha Dias S, Hayward R, Savage K, Delmas V, et al. Oncogenic BRAF induces melanocyte senescence and melanoma in mice. *Cancer Cell* 2009;15:294–303. [S1535-6108\(09\)00074-9](https://doi.org/10.1016/j.ccr.2009.02.022) [pii]10.1016/j.ccr.2009.02.022.
  - [93] Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci USA* 2008;105:3041–6. <http://dx.doi.org/10.1073/pnas.0711741105>.
  - [94] Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* 2010;363:809–19. <http://dx.doi.org/10.1056/NEJMoa1002011>.
  - [95] Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 2011;364:2507–16. <http://dx.doi.org/10.1056/NEJMoa1103782>.
  - [96] Hyman DM, Puzanov I, Subbiah V, Faris JE, Chau I, Blay J-Y, et al. Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. *N Engl J Med* 2015;373:726–36. <http://dx.doi.org/10.1056/NEJMoa1502309>.
  - [97] Hall JM, Lee MK, Newman B, Morrow JE, Anderson LA, Huey B, et al. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 1990;250:1684–9.
  - [98] Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66–71.
  - [99] Wooster R, Neuhausen SL, Mangion J, Quirk Y, Ford D, Collins N, et al. Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12–13. *Science* 1994;265:2088–90.
  - [100] Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, et al. Identification of the breast cancer susceptibility gene BRCA2. *Nature* 1995;378:789–92. <http://dx.doi.org/10.1038/378789a0>.
  - [101] Burke W, Daly M, Garber J, Botkin J, Kahn MJ, Lynch P, et al. Recommendations for follow-up care of individuals with an inherited predisposition to cancer. II. BRCA1 and BRCA2. *Cancer Genetics Studies Consortium. JAMA* 1997;277:997–1003.
  - [102] Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005;434:913–7. <http://dx.doi.org/10.1038/nature03443>.
  - [103] Farmer H, McCabe N, Lord CJ, Tutt ANJ, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005;434:917–21. <http://dx.doi.org/10.1038/nature03445>.
  - [104] Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* 2009;361:123–34. <http://dx.doi.org/10.1056/NEJMoa0900212>.
  - [105] Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. *Lancet Oncol* 2014;15:852–61. [http://dx.doi.org/10.1016/S1470-2045\(14\)70228-1](http://dx.doi.org/10.1016/S1470-2045(14)70228-1).
  - [106] Cobleigh MA, Langmuir VK, Sledge GW, Miller KD, Haney L, Novotny WF, et al. A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. *Semin Oncol* 2003;30:117–24. doi: [S0093775403004469](https://doi.org/10.1053/S0093775403004469) [pii].
  - [107] Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;349:427–34.
  - [108] van der Veldt A, Meijerink MR, van den Eertwegh AJM, Boven E. Targeted therapies in renal cell cancer: recent developments in imaging. *Target. Oncol* 2010;5:95–112. <http://dx.doi.org/10.1007/s11523-010-0146-5>.
  - [109] Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* 2009;15:7412–20. doi: [1078-0432.CCR-09-1624](https://doi.org/10.1158/1078-0432.CCR-09-1624) [pii]10.1158/1078-0432.CCR-09-1624.
  - [110] Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010;363:411–22.
  - [111] Workman P. Auditing the pharmacological accounts for Hsp90 molecular chaperone inhibitors: unfolding the relationship between pharmacokinetics and pharmacodynamics. *Mol Cancer Ther* 2003;2:131–8.
  - [112] Yap TA, Sandhu SK, Workman P, de Bono JS. Envisioning the future of early anticancer drug development. *Nat Rev Cancer* 2010;10:514–23. <http://dx.doi.org/10.1038/nrc2870>.
  - [113] Mullard A. Learning from exceptional drug responders. *Nat Rev Drug Discov* 2014;13:401–2. <http://dx.doi.org/10.1038/nrd4338>.
  - [114] Perez-Gracia JL, Ruiz-Ilundain MG, Garcia-Ribas I, Carrasco EM. The role of extreme phenotype selection studies in the identification of clinically relevant genotypes in cancer research. *Cancer* 2002;95:1605–10.
  - [115] Perez-Gracia JL, Gurrpide A, Ruiz-Ilundain MG, Alfaro Alegria C, Colomer R, Garcia-Foncillas J, et al. Selection of extreme phenotypes: the role of clinical observation in translational research. *Clin Transl Oncol* 2010;12:174–80. [CLAT360](https://doi.org/10.1038/clnt.2010.12) [pii].
  - [116] Emond MJ, Louie T, Emerson J, Zhao W, Mathias RA, Knowles MR, et al. Exome sequencing of extreme phenotypes identifies DCTN4 as a modifier of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis. *Nat Genet* 2012;44:886–9. [ng.2344](https://doi.org/10.1038/ng.2344) [pii]10.1038/ng.2344.
  - [117] Iyer G, Hanrahan AJ, Milowsky MI, Al-Ahmadie H, Scott SN, Janakiraman M, et al. Genome sequencing identifies a basis for everolimus sensitivity. *Science* 2012;338:221. <http://dx.doi.org/10.1126/science.1226344>.
  - [118] Wagle N, Grabiner BC, Van Allen EM, Amin-Mansour A, Taylor-Weiner A, Rosenberg M, et al. Response and acquired resistance to everolimus in anaplastic thyroid cancer. *N Engl J Med* 2014;371:1426–33. <http://dx.doi.org/10.1056/NEJMoa1403352>.
  - [119] Wagle N, Grabiner BC, Van Allen EM, Hodis E, Jacobus S, Supko JG, et al. Activating mTOR mutations in a patient with an extraordinary response on a phase I trial of everolimus and pazopanib. *Cancer Discov* 2014;4:546–53. <http://dx.doi.org/10.1158/2159-8290.CD-13-0353>.
  - [120] Van Allen EM, Mouw KW, Kim P, Iyer G, Wagle N, Al-Ahmadie H, et al. Somatic ERCC2 mutations correlate with cisplatin sensitivity in muscle-invasive urothelial carcinoma. *Cancer Discov* 2014;4:1140–53. <http://dx.doi.org/10.1158/2159-8290.CD-14-0623>.
  - [121] Tuchman M, Stoeckeler JS, Kiang DT, O'Dea RF, Ramnaraine ML, Mirkin BL. Familial pyrimidinemia and pyrimidinuria associated with severe fluorouracil toxicity. *N Engl J Med* 1985;313:245–9.
  - [122] Van Kuilenburg AB, Vreken P, Beex LV, Meinsma R, Van Lenthe H, De Abreu RA, et al. Heterozygosity for a point mutation in an invariant splice donor site of dihydropyrimidine dehydrogenase and severe 5-fluorouracil related toxicity. *Eur J Cancer* 1997;33:2258–64.
  - [123] Ando Y, Saka H, Ando M, Sawa T, Muro K, Ueoka H, et al. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. *Cancer Res* 2000;60:6921–6.
  - [124] Perez-Gracia JL, Pajares MJ, Andueza MP, Pita G, de Torres Tajes JP, Casanova C, et al. Identification through genome-wide association study (GWAS) of single nucleotide polymorphisms (SNPs) associated with extreme phenotypes of tobacco-induced non-small cell lung cancer (NSCLC) risk. *ASCO Meet Abstr* 2014;32:11046.
  - [125] Perez-Gracia JL, Ruiz-Ilundain MG. Cancer protective mutations: looking for the needle in the haystack. *Clin Transl Oncol* 2001;3:169–71.
  - [126] Emond MJ, Louie T, Emerson J, Chong JX, Mathias RA, Knowles MR, et al. Exome sequencing of phenotypic extremes identifies CAV2 and TMC6 as interacting modifiers of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis. *PLoS Genet* 2015;11:e1005273. <http://dx.doi.org/10.1371/journal.pgen.1005273>.
  - [127] Hardy J, Singleton A. Genomewide association studies and human disease. *N Engl J Med* 2009;360:1759–68. <http://dx.doi.org/10.1056/NEJMr0808700>.
  - [128] Ghosh D, Poisson LM. "Omics" data and levels of evidence for biomarker discovery. *Genomics* 2009;93:13–6. <http://dx.doi.org/10.1016/j.ygeno.2008.07.006>.

- [129] Guo Y, Graber A, McBurney RN, Balasubramanian R. Sample size and statistical power considerations in high-dimensionality data settings: a comparative study of classification algorithms. *BMC Bioinformatics* 2010;11:447. <http://dx.doi.org/10.1186/1471-2105-11-447>.
- [130] Chi Y-Y, Gribbin MJ, Johnson JL, Muller KE. Power calculation for overall hypothesis testing with high-dimensional commensurate outcomes. *Stat Med* 2014;33:812–27. <http://dx.doi.org/10.1002/sim.5986>.
- [131] Guo Y, Zhao S, Li C-I, Sheng Q, Shyr Y. RNAseqPS: a web tool for estimating sample size and power for RNAseq experiment. *Cancer Inform* 2014;13:1–5. <http://dx.doi.org/10.4137/CIN.S17688>.
- [132] Ching T, Huang S, Garmire LX. Power analysis and sample size estimation for RNA-Seq differential expression. *RNA* 2014;20:1684–96. <http://dx.doi.org/10.1261/rna.046011.114>.
- [133] Simon R. Design and analysis of clinical trials. In: DeVita, Hellman, Rosenberg's Cancer Princ. Pract. Oncol. Philadelphia: Wolters Kluwer Health-Lippincott Williams & Wilkins; 2015. p. 398–415.
- [134] Hewitt SM, Lewis FA, Cao Y, Conrad RC, Cronin M, Danenberg KD, et al. Tissue handling and specimen preparation in surgical pathology: issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue. *Arch Pathol Lab Med* 2008;132:1929–35. <http://dx.doi.org/10.1043/1543-2165-132.12.1929>.
- [135] Sandusky G, Dumaual C, Cheng L. Review paper: Human tissues for discovery biomarker pharmaceutical research: the experience of the Indiana University Simon Cancer Center-Lilly Research Labs Tissue/Fluid BioBank. *Vet Pathol* 2009;46:2–9. <http://dx.doi.org/10.1354/vp.46-1-2>.
- [136] Fitzgibbons PL, Bradley LA, Fatheree LA, Alsabeh R, Fulton RS, Goldsmith JD, et al. Principles of analytic validation of immunohistochemical assays: Guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med* 2014;138:1432–43. <http://dx.doi.org/10.5858/arpa.2013-0610-CP>.
- [137] Economou M, Schöni L, Hammer C, Galván JA, Mueller D-E, Zlobec I. Proper paraffin slide storage is crucial for translational research projects involving immunohistochemistry stains. *Clin Transl Med* 2014;3:4. <http://dx.doi.org/10.1186/2001-1326-3-4>.
- [138] Oki M, Saka H, Kitagawa C, Kogure Y, Murata N, Adachi T, et al. Rapid on-site cytologic evaluation during endobronchial ultrasound-guided transbronchial needle aspiration for diagnosing lung cancer: a randomized study. *Respiration* 2013;85:486–92. <http://dx.doi.org/10.1159/000346987>.
- [139] Douillard J-Y, Ostoros G, Cobo M, Ciuleanu T, McCormack R, Webster A, et al. First-line gefitinib in Caucasian EGFR mutation-positive NSCLC patients: a phase-IV, open-label, single-arm study. *Br J Cancer* 2014;110:55–62. <http://dx.doi.org/10.1038/bjc.2013.721>.
- [140] Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Roeser JC, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *N Engl J Med* 2014;371:1028–38. <http://dx.doi.org/10.1056/NEJMoa1315815>.
- [141] Lohr JG, Adalsteinsson VA, Cibulskis K, Choudhury AD, Rosenberg M, Cruz-Gordillo P, et al. Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nat Biotechnol* 2014;32:479–84. <http://dx.doi.org/10.1038/nbt.2892>.
- [142] Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012;486:532–6. <http://dx.doi.org/10.1038/nature11156>.
- [143] Oxnard GR, Pawletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res* 2014;20:1698–705. <http://dx.doi.org/10.1158/1078-0432.CCR-13-2482>.
- [144] Sanmamed MF, Fernandez-Landazuri S, Rodriguez C, Zarate R, Lozano MD, Zubiri L, et al. Quantitative cell-free circulating BRAFV600E mutation analysis by use of droplet digital PCR in the follow-up of patients with melanoma being treated with BRAF inhibitors. *Clin Chem* 2015;61:297–304. <http://dx.doi.org/10.1373/clinchem.2014.230235>.
- [145] Sanmamed MF, Carranza-Rua O, Alfaro C, Oñate C, Martín-Algarra S, Perez G, et al. Serum interleukin-8 reflects tumor burden and treatment response across malignancies of multiple tissue origins. *Clin Cancer Res* 2014;20:5697–707. <http://dx.doi.org/10.1158/1078-0432.CCR-13-3203>.
- [146] Diaz LA, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 2012;486:537–40. <http://dx.doi.org/10.1038/nature11219>.
- [147] Bettgeowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;6:224ra24. <http://dx.doi.org/10.1126/scitranslmed.3007094>.
- [148] Ajona D, Pajares MJM, Corrales L, Perez-Gracia JL, Agorreta J, Lozano MD, et al. Investigation of complement activation product c4d as a diagnostic and prognostic biomarker for lung cancer. *J Natl Cancer Inst* 2013;105:1385–93. <http://dx.doi.org/10.1093/jnci/djt205>.
- [149] Harmon A. New Drugs Stir Debate on Rules of Clinical Trials; 2010. <[http://www.nytimes.com/2010/09/19/health/research/19trial.html?ref=target\\_cancer](http://www.nytimes.com/2010/09/19/health/research/19trial.html?ref=target_cancer)>.
- [150] Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009;361:958–67. <http://dx.doi.org/10.1056/NEJMoa0904554>.
- [151] D'Addario G, Felip E. ESMO Guidelines Working Group. Non-small-cell lung cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol* 2008;19(Suppl 2):ii39–40. <http://dx.doi.org/10.1093/annonc/mdn081>.
- [152] NCI-Molecular Analysis for Therapy Choice (NCI-MATCH) Trial – National Cancer Institute n.d. <http://www.cancer.gov/about-cancer/treatment/clinical-trials/nci-supported/nci-match> (accessed May 3, 2016).
- [153] Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366:2443–54. <http://dx.doi.org/10.1056/NEJMoa1200690>.
- [154] Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 2014;371. <http://dx.doi.org/10.1056/NEJMoa1406498>.
- [155] Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015;348:124–8. <http://dx.doi.org/10.1126/science.1257631>.
- [156] McGranahan N, Furness AJS, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science* 2016. <http://dx.doi.org/10.1126/science.1257631>.
- [157] Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJM, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014;515:568–71. <http://dx.doi.org/10.1038/nature13954>.
- [158] Powles T, Eder JP, Fine GD, Braiteh FS, Lortie Y, Cruz C, et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* 2014;515:558–62. <http://dx.doi.org/10.1038/nature13904>.
- [159] Fusi A, Festino L, Botti G, Masucci G, Melero I, Lorigan P, et al. PD-L1 expression as a potential predictive biomarker. *Lancet Oncol* 2015;16:1285–7. [http://dx.doi.org/10.1016/S1470-2045\(15\)00307-1](http://dx.doi.org/10.1016/S1470-2045(15)00307-1).
- [160] Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* 2013;39:1–10. <http://dx.doi.org/10.1016/j.immuni.2013.07.012>.
- [161] Mlecnik B, Bindea G, Kirilovsky A, Angell HK, Obenauf AC, Tosolini M, et al. The tumor microenvironment and Immunoscore are critical determinants of dissemination to distant metastasis. *Sci Transl Med* 2016;8:327ra26. <http://dx.doi.org/10.1126/scitranslmed.aad6352>.
- [162] Melero I, Berman DM, Aznar MA, Korman AJ, Pérez Gracia JL, Haanen J. Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nat Rev Cancer* 2015;15:457–72. <http://dx.doi.org/10.1038/nrc3973>.
- [163] Frohling S, Scholl C, Levine RL, Loriaux M, Boggon TJ, Bernard OA, et al. Identification of driver and passenger mutations of FLT3 by high-throughput DNA sequencing analysis and functional assessment of candidate alleles. *Cancer Cell* 2007;12:501–13. doi: S1535-6108(07)00336-4 [pii]10.1016/j.ccr.2007.11.005.
- [164] Bivona TG, Hieronymus H, Parker J, Chang K, Taron M, Rosell R, et al. FAS and NF-κB signalling modulate dependence of lung cancers on mutant EGFR. *Nature* 2011;471:523–6. <http://dx.doi.org/10.1038/nature09870>.
- [165] Crystal AS, Shaw AT, Sequist LV, Fribolet L, Niederst MJ, Lockerman EL, et al. Patient-derived models of acquired resistance can identify effective drug combinations for cancer. *Science* 2014;346:1480–6. <http://dx.doi.org/10.1126/science.1254721>.
- [166] Lin L, Sabnis AJ, Chan E, Olivas V, Cade L, Pazarentzos E, et al. The Hippo effector YAP promotes resistance to RAF- and MEK-targeted cancer therapies. *Nat Genet* 2015;47:250–6. <http://dx.doi.org/10.1038/ng.3218>.
- [167] Hrustanovic G, Olivas V, Pazarentzos E, Tulpule A, Asthana S, Blakely CM, et al. RAS-MAPK dependence underlies a rational polytherapy strategy in EML4-ALK-positive lung cancer. *Nat Med* 2015;21:1038–47. <http://dx.doi.org/10.1038/nm.3930>.
- [168] van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530–6.
- [169] Ma XJ, Salunga R, Tuggle JT, Gaudet J, Enright E, McQuary P, et al. Gene expression profiles of human breast cancer progression. *Proc Natl Acad Sci USA* 2003;100:5974–9.
- [170] Paik S, Shak S, Tang C, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med* 2004;351:2817–26.
- [171] Ayers M, Symmans WF, Stec J, Damokosh AI, Clark E, Hess K, et al. Gene expression profiles predict complete pathologic response to neoadjuvant paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide chemotherapy in breast cancer. *J Clin Oncol* 2004;22:2284–93. <http://dx.doi.org/10.1200/JCO.2004.05.1661>.
- [172] Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci USA* 2003;100:9440–5. <http://dx.doi.org/10.1073/pnas.1530509100>.
- [173] Hastie T, Tibshirani R, Friedman J. The elements of statistical learning: data mining, inference, and prediction. 2nd ed. New York: Springer-Verlag; 2009. <http://dx.doi.org/10.1007/978-0-387-84858-7>.
- [174] Renshaw HW, Varghese RS, Zhang Z, Xuan J, Clarke R. Classification algorithms for phenotype prediction in genomics and proteomics. *Front Biosci* 2008;13:691–708.
- [175] Kantardzic M. Data mining: concepts, models, methods, and algorithms. Hoboken: John Wiley & Sons, Inc.; 2011. <http://dx.doi.org/10.1002/9781118029145.fmatter>.
- [176] Sham PC, Purcell SM. Statistical power and significance testing in large-scale genetic studies. *Nat Rev Genet* 2014;15:335–46. <http://dx.doi.org/10.1038/nrg3706>.



- [177] Li D, Lewinger JP, Gauderman WJ, Murcray CE, Conti D. Using extreme phenotype sampling to identify the rare causal variants of quantitative traits in association studies. *Genet Epidemiol* 2011;35:790–9. <http://dx.doi.org/10.1002/gepi.20628>.
- [178] Food and Drug Administration. Paving the Way for Personalized Medicine; 2013. <<http://www.fda.gov/scienceresearch/specialtopics/personalizedmedicine/default.htm>> (accessed October 3, 2015).
- [179] Fitzpatrick PE, McKenzie KD, Beasley A, Sheehan JD. Patients attending tertiary referral urology clinics: willingness to participate in tissue banking. *BJU Int* 2009;104:209–13. <http://dx.doi.org/10.1111/j.1464-410X.2009.08666.x>.
- [180] McGuire WL, Carbone PP, Sears ME, Vollmer EP. Estrogen receptors in human breast cancer: an overview. In: *Estrogen Recept. Hum. Breast Cancer*. New York: Raven Press; 1974. p. 1–8.
- [181] Kalinsky K, Mayer JA, Xu X, Pham T, Wong KL, Villarin E, et al. Correlation of hormone receptor status between circulating tumor cells, primary tumor, and metastasis in breast cancer patients. *Clin Transl Oncol* 2015;17:539–46. <http://dx.doi.org/10.1007/s12094-015-1275-1>.
- [182] Colomer R, Montero S, Lluh A, Ojeda B, Barnadas A, Casado A, et al. Circulating HER2 extracellular domain and resistance to chemotherapy in advanced breast cancer. *Clin Cancer Res* 2000;6:2356–62.
- [183] Kostler WJ, Schwab B, Singer CF, Neumann R, Rucklinger E, Brodowicz T, et al. Monitoring of serum Her-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer. *Clin Cancer Res* 2004;10:1618–24.
- [184] Kang G, Bae BN, Sohn BS, Pyo J-S, Kang GH, Kim K-M. Detection of KIT and PDGFR mutations in the plasma of patients with gastrointestinal stromal tumor. *Target Oncol* 2015. <http://dx.doi.org/10.1007/s11523-015-0361-1>.
- [185] Maheswaran S, Sequist LV, Nagrath S, Ullkus L, Brannigan B, Collura CV, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;359:366–77. doi: [10.1056/NEJMoa0800668](https://doi.org/10.1056/NEJMoa0800668) [pii]10.1056/NEJMoa0800668.
- [186] Ilie M, Long E, Butori C, Hofman V, Coelle C, Mauro V, et al. ALK-gene rearrangement: a comparative analysis on circulating tumour cells and tumour tissue from patients with lung adenocarcinoma. *Ann Oncol* 2012;23:2907–13. <http://dx.doi.org/10.1093/annonc/mds137>.
- [187] Yen LC, Yeh YS, Chen CW, Wang HM, Tsai HL, Lu CY, et al. Detection of KRAS oncogene in peripheral blood as a predictor of the response to cetuximab plus chemotherapy in patients with metastatic colorectal cancer. *Clin Cancer Res* 2009;15:4508–13. doi: [1078-0432.CCR-08-3179](https://doi.org/10.1158/1078-0432.CCR-08-3179) [pii]10.1158/1078-0432.CCR-08-3179.
- [188] Shinozaki M, O'Day SJ, Kitago M, Amersi F, Kuo C, Kim J, et al. Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. *Clin Cancer Res* 2007;13:2068–74. doi: [13/7/2068](https://doi.org/10.1158/1078-0432.CCR-06-2120) [pii]10.1158/1078-0432.CCR-06-2120.
- [189] Doebele RC, Davis LE, Vaishnavi A, Le AT, Estrada-Bernal A, Keysar S, et al. An oncogenic NTRK fusion in a soft tissue sarcoma patient with response to the tropomyosin-related kinase (TRK) inhibitor LOXO-101. *Cancer Discov* 2015;5:1049–57. <http://dx.doi.org/10.1158/2159-8290.CD-15-0443>.
- [190] Van Allen EM, Lui VVWY, Egloff AM, Goetz EM, Li H, Johnson JT, et al. Genomic Correlate of Exceptional Erlotinib Response in Head and Neck Squamous Cell Carcinoma. *JAMA Oncol* 2015;1:238–44. <http://dx.doi.org/10.1001/jamaoncol.2015.34>.
- [191] Cools J, DeAngelo DJ, Gotlib J, Stover EH, Legare RD, Cortes J, et al. A tyrosine kinase created by fusion of the PDGFR and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med* 2003;348:1201–14. <http://dx.doi.org/10.1056/NEJMoa025217>.
- [192] Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:e73. doi: [10.1371/journal.pmed.0020073](https://doi.org/10.1371/journal.pmed.0020073) [pii]10.1371/journal.pmed.0020073.
- [193] Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010;363:1734–9. <http://dx.doi.org/10.1056/NEJMoa1007478>.
- [194] Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B. Mechanisms of acquired crizotinib resistance in ALK-rearranged lung Cancers. *Sci Transl Med* 2012;4:120ra17. <http://dx.doi.org/10.1126/scitranslmed.3003316>.
- [195] Shaw AT, Friboulet L, Leshchiner I, Gainor JF, Bergqvist S, Brooun A, et al. Resensitization to Crizotinib by the Lorlatinib ALK Resistance Mutation L1198F. *N Engl J Med* 2016;374:54–61. <http://dx.doi.org/10.1056/NEJMoa1508887>.
- [196] Awad MM, Katayama R, McTigue M, Liu W, Deng YL, Brooun A, et al. Acquired resistance to crizotinib from a mutation in CD74-ROS1. *N Engl J Med* 2013;368:2395–401. <http://dx.doi.org/10.1056/NEJMoa1215530>.
- [197] Emery CM, Vijayendran KG, Zipser MC, Sawyer AM, Niu L, Kim JJ, et al. MEK1 mutations confer resistance to MEK and B-RAF inhibition. *Proc Natl Acad Sci USA* 2009;106:20411–6. <http://dx.doi.org/10.1073/pnas.0905833106>.
- [198] Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* 2010;468:968–72. <http://dx.doi.org/10.1038/nature09627>.
- [199] Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* 2010;468:973–7. <http://dx.doi.org/10.1038/nature09626>.
- [200] Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol* 2011;29:3085–96. <http://dx.doi.org/10.1200/JCO.2010.33.2312>.
- [201] Poulikakos PI, Persaud Y, Janakiraman M, Kong X, Ng C, Moriceau G, et al. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF (V600E). *Nature* 2011;480:387–90. <http://dx.doi.org/10.1038/nature10662>.
- [202] Shi H, Moriceau G, Kong X, Lee M-K, Lee H, Koya RC, et al. Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat Commun* 2012;3:724. <http://dx.doi.org/10.1038/ncomms1727>.
- [203] Trunzer K, Pavlick AC, Schuchter L, Gonzalez R, McArthur GA, Hutson TE, et al. Pharmacodynamic effects and mechanisms of resistance to vemurafenib in patients with metastatic melanoma. *J Clin Oncol* 2013;31:1767–74. <http://dx.doi.org/10.1200/JCO.2012.44.7888>.
- [204] Van Allen EM, Wagle N, Sucker A, Treacy DJ, Johannessen CM, Goetz EM, et al. The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer Discov* 2014;4:94–109. <http://dx.doi.org/10.1158/2159-8290.CD-13-0617>.
- [205] Ahronian LG, Sennott EM, Van Allen EM, Wagle N, Kwak EL, Faris JE, et al. Clinical acquired resistance to RAF inhibitor combinations in BRAF-mutant colorectal cancer through MAPK pathway alterations. *Cancer Discov* 2015;5:358–67. <http://dx.doi.org/10.1158/2159-8290.CD-14-1518>.
- [206] Debiec-Rychter M, Cools J, Dumez H, Sciot R, Stul M, Mentens N, et al. Mechanisms of resistance to imatinib mesylate in gastrointestinal stromal tumors and activity of the PKC412 inhibitor against imatinib-resistant mutants. *Gastroenterology* 2005;128:270–9. <http://dx.doi.org/10.1053/j.gastro.2004.11.020>.
- [207] Wardelmann E, Merkelbach-Bruse S, Pauls K, Thomas N, Schildhaus H-U, Heinicke T, et al. Polyclonal evolution of multiple secondary KIT mutations in gastrointestinal stromal tumors under treatment with imatinib mesylate. *Clin Cancer Res* 2006;12:1743–9. <http://dx.doi.org/10.1158/1078-0432.CCR-05-1211>.
- [208] Lim K-H, Huang M-J, Chen L-T, Wang T-E, Liu C-L, Chang C-S, et al. Molecular analysis of secondary kinase mutations in imatinib-resistant gastrointestinal stromal tumors. *Med Oncol* 2008;25:207–13. <http://dx.doi.org/10.1007/s12032-007-9014-2>.
- [209] Liegl B, Kepten I, Le C, Zhu M, Demetri GD, Heinrich MC, et al. Heterogeneity of kinase inhibitor resistance mechanisms in GIST. *J Pathol* 2008;216:64–74. <http://dx.doi.org/10.1002/path.2382>.
- [210] Tamborini E, Bonadiman L, Greco A, Albertini V, Negri T, Gronchi A, et al. A new mutation in the KIT ATP pocket causes acquired resistance to imatinib in a gastrointestinal stromal tumor patient. *Gastroenterology* 2004;127:294–9.
- [211] Serrano C, Wang Y, Mariño-Enríquez A, Lee J-C, Ravegnini G, Morgan JA, et al. KRAS and KIT gatekeeper mutations confer polyclonal primary imatinib resistance in GI stromal tumors: relevance of concomitant phosphatidylinositol 3-kinase/AKT dysregulation. *J Clin Oncol* 2015;33:e93–396. <http://dx.doi.org/10.1200/JCO.2013.48.7488>.
- [212] Zaretsky JM, Garcia-Diaz A, Shin DS, Escuin-Ordinas H, Hugo W, Hu-Lieskovan S, et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. *N Engl J Med* 2016;375:819–29. <http://dx.doi.org/10.1056/NEJMoa1604958>.
- [213] Garcia-Donas J, Esteban E, Leandro-García LJ, Castellano DE, del Alba AG, Clinet MA, et al. Single nucleotide polymorphism associations with response and toxic effects in patients with advanced renal-cell carcinoma treated with first-line sunitinib: a multicentre, observational, prospective study. *Lancet Oncol* 2011;12:1143–50. [http://dx.doi.org/10.1016/S1470-2045\(11\)70266-2](http://dx.doi.org/10.1016/S1470-2045(11)70266-2).
- [214] Prior C, Perez-Gracia JL, Garcia-Donas J, Rodríguez-Antona C, Guruceaga E, Esteban E, et al. Identification of tissue microRNAs predictive of sunitinib activity in patients with metastatic renal cell carcinoma. *PLoS One* 2014;9:e86263. <http://dx.doi.org/10.1371/journal.pone.0086263>.
- [215] Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med* 2000;343:1350–4. <http://dx.doi.org/10.1056/NEJM200011093431901>.