
Title: Molecular Testing in Oncology: Problems, Pitfalls and Progress

Author: Cathal P. O'Brien Sarah E. Taylor John J. O'Leary Stephen P. Finn

PII: S0169-5002(13)00580-1
DOI: http://dx.doi.org/doi:10.1016/j.lungcan.2013.12.010
Reference: LUNG 4507

To appear in: *Lung Cancer*

Received date: 11-12-2013
Accepted date: 21-12-2013

Please cite this article as: O'Brien CP, Taylor SE, O'Leary JJ, Finn SP, Molecular Testing in Oncology: Problems, Pitfalls and Progress, *Lung Cancer* (2014), http://dx.doi.org/10.1016/j.lungcan.2013.12.010

*Invited Review*

# Molecular Testing in Oncology: Problems, Pitfalls and Progress

Cathal P O'Brien[1,2,3], Sarah E Taylor[4], John J O'Leary[2.3,5], Stephen P Finn[1,2,3]

[1]*Cancer Molecular Diagnostics, St James's Hospital, Dublin 8, Ireland*

[2]*Department of Histopathology, St James's Hospital, Dublin 8, Ireland*

[3]*Department of Histopathology, Trinity College Dublin, Dublin 8, Ireland*

[4] Department of Obstetrics and Gynecology, Magee-Womens Hospital, University of Pittsburgh Medical Center, 300 Halket Street, Pittsburgh, PA, 15213, USA

[5]*Department of Histopathology, The Coombe Women and Children's Hospital, Dublin 8, Ireland*

**Key words:**

**Corresponding author:**

Dr Cathal O'Brien

Cancer Molecular Diagnostics

Central Pathology Laboratory

St. James's Hospital

Dublin 8

Ireland

Tel: 353 1 4103567

Email: obriec12@tcd.ie

**Abstract**

Recent advances in the understanding of the molecular basis of cancer and the development of molecular diagnostics based on this knowledge have done much to progress the fields of oncology and pathology. Technological developments such as Next Generation Sequencing (NGS) and multiplex assays have made feasible the widespread adoption of molecular diagnostics for clinical use. While these developments and advances carry much promise, there are pitfalls to implementing this testing.

Choosing appropriate biomarkers is a vital first step for clinical use and being able to understand the complex relationship between predictive and prognostic biomarkers is a crucial component of this. Testing for standard of care biomarkers is not straightforward, one must choose carefully between clinical trial assays, assays that analyse the same biological phenomenon or surrogate biomarkers. Sample heterogeneity and population specific difference is assays may skew results and must be controlled for at the assay design stage.

At a technical level, NGS has the potential to revolutionise laboratory practice and approaches to cancer treatment. However, use of this technology requires careful planning and implementation if one is to avoid technical and ethical quagmires. Finally, with FDA regulation of companion diagnostics one may be limited to therapy specific assays.

## Introduction

If one casts a broad net, molecular diagnostics have featured in the pathologic assessment of cancer since the advent of immunohistochemistry (IHC). The ability of IHC to selectively stain specific protein molecules for subsequent microscopic evaluation merits classification as a molecular technique. Likewise, the ability of in-situ hybridization (ISH) to identify cancer associated abnormalities at the chromosomal or transcriptomic levels similarly falls into this category. Thus, the recent development of mandatory treatment-guiding biomarker assays primarily using PCR based techniques for solid tumours, including lung cancer, marks an addition to the pathology toolset rather than a fresh departure from the morphological roots of pathology.

Traditionally, the role of the pathologist was to diagnose disease and determine prognosis based on the macroscopic and microscopic appearance of tissue. In the setting of cancer diagnosis, the pathologist not only identifies a lesion as malignant, but also suggests whether the lesion may have arisen in situ or is likely to be metastatic. Thus, this information is used for staging and guiding treatment based on the broad classification of the tumour. More recently, identifying the tumour histologic sub-type has shown to be predictive of response to certain types of therapy. A study by Scagliotti *et al* [1], showed that in lung cancer histologic sub-type predicted response to one of two cisplatin doublet therapies. Patients with lung adenocarcinoma were shown to have a greater overall survival when prescribed cisplatin/pemetrexed versus cisplatin/gemcitabine. Conversely, patients with squamous cell histology

demonstrated a better overall survival when prescribed cisplatin/gemcitabine versus cisplatin/pemetrexed.

Advances in our understanding of the molecular basis of cancer have lead to the development of targeted therapies, such as trastuzumab for the treatment of HER-2 overexpressing metastatic breast cancer [2] and imatinib for the treatment of chronic myelogenous leukaemia [3] and gastrointestinal stromal tumours [4]. Both of these early developments demonstrated that a targeted approach could yield significant survival benefits provided the patient's malignancy contained a molecular defect that could be specifically targeted with inhibitor therapy. Thus, targeted therapy has necessitate molecular assays to identify specific aberrations that may indicate or contraindicate a given therapy.

While molecular testing of tumours is of undeniable benefit, it is not without its pitfalls. This article will explore key areas that are sources of confusion or misinterpretation in molecular testing with a particular focus on predictive and prognostic biomarkers. Laboratory and clinical features that may cause pre-analytical and analytical errors such as sample mix-ups, processing considerations and PCR contamination are also well described in other literature sources [5–7] and are beyond the scope of this article. For the purposes of this review, those sources of uncertainty and confusion that are specific to molecular testing for oncology will be discussed and where possible, potential solutions for these issues will be presented. As the technology and knowledge supporting molecular testing is rapidly evolving, the advantages and challenges of these developments will also be discussed.

**Not all biomarkers are clinically relevant**

It is important to recognise that not all biomarkers are created equal and very few potential biomarkers live up to the standard required for clinical implementation. For predictive markers considered to be companion diagnostics, i.e. those that are used for patient stratification for a clinical trial, the trial itself should provide sufficient evidence for use of the biomarker in a clinical setting. A well known example of this level of evidence is found in the **I**RESSA **P**an-**AS**ia **S**tudy (IPASS) trial [8]. This trial demonstrated that patients with an *EGFR* positive mutation test had a longer progression free survival (PFS) if prescribed gefitinib versus carboplatin plus paclitaxel. Conversely, patients with a negative EGFR mutation test had shorter PFS when prescribed gefitinib versus carboplatin plus paclitaxel. The clearly defined nature of the biomarker in this trial and a clear understanding of the underlying biology [9] has lead to its adoption as a marker of treatment suitability for gefitinib [10,11].

Currently, EGFR mutation testing and ALK rearrangement status by break-apart FISH assay are the only two molecular markers considered standard of care for Non-Small Cell Lung Carcinoma (NSCLC) treatment and are the subject of the College of American Pathologists (CAP), International Association for Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) Guidelines published in 2013 [12]. *EGFR* mutation and *ALK* rearrangement testing are also a feature of the Version 1.2014 National Comprehensive Cancer Network (NCCN) clinical practice guidelines for Non-Small Cell Lung Carcinoma [11]. The 1.2014 version of the NCCN NSCLC guidelines highlight two additional markers for possible implementation in treatment pathways; ERCC1 expression levels as a prognostic marker and predictor of response to platinum based chemotherapies

[13–15] and *KRAS* mutation testing as a potential prognostic marker for NSCLC [16,17], although the latter link is not universally supported [18].

To some, the relative paucity of the previously mentioned molecular markers of NSCLC prognosis seems a little surprising, as numerous other biomarkers have been suggested to be important in the management of NSCLC. Frequently, novel technologies can result in biomarkers that show great promise but fail to live up to the standards required for clinical decision-making. As an example, early microarray studies aimed at solving prognostic dilemmas in early stage lung cancer were suggested to provide clinically relevant prognostic information by study authors. However, a review of 16 published microarray studies found that none demonstrated evidence of suitability for clinical use [19]. Established techniques may also be prone to early promise and poor results; A review of IHC antibodies tested for prognostic value in NSCLC failed to identify any single or combined marker that provided sufficient prognostic information to be clinically useful [20].

As a level of uncertainty exists regarding which markers to use in molecular pathology, it is always best to operate with those supported by best practice guidelines. One could easily cite hundreds for articles which promote the utility of a novel marker in lung cancer, or any other cancer for that matter. Most importantly, the chosen markers should be supported by research sufficient to give confidence in the marker's ability to deliver a clinically meaningful result. One need only review the chequered history of ERCC1 testing to realise the importance of prospective trials in establishing the utility of a biomarker. Currently, testing of a broad range of markers is more suited to clinical trials

than routine practice. Trials such as the SHIVA trial will aim to assess the clinical utility of emerging markers for targeted therapies [21].

**Standard-of-care versus surrogate markers**

In pathology laboratories, certain technologies are favoured over others due to their widespread use and availability. A prime example of this is automated immunohistochemistry (IHC), which is available in nearly every histopathology laboratory. Both pathologists and laboratory scientists are familiar with IHC and dialog around the subject is facilitated by this experience. Within laboratories that do not routinely perform PCR or FISH testing, there is an inclination to favour IHC-based testing. However, this rationale may lead to more problems than solutions as outlined below.

Detection of *ALK* translocations in lung cancer is predictive of response to crizotinib therapy [22]. In a clinical trial that compared crizotinib versus chemotherapy in advanced *ALK* translocation positive lung cancer, *ALK* break-apart FISH was used to demonstrate the presence of a translocation in the sample and select the patient for inclusion in the trial. Break-apart FISH requires the counting of a set proportion of cells in which a split signal, or split deleted signal i.e. a non proximal 5' and 3' *ALK* probe is identified [23]. Analytically, the break-apart FISH assay does not identify specific *ALK* fusions, rather it detects a break in the chromosomal region encoding the ALK tyrosine kinase domain. This assay can detect rarer translocations in which *ALK* is paired with a different fusion partner such as *KIF5B* [24], *KLC1* [25] or others. Thus, the inclusion criteria for clinical trials based upon the break-apart FISH assay is the presence of an *ALK* rearrangement. It is self-evident that assays which seek to act as

alternatives to that used in the clinical trial would need to detect the same phenomenon (i.e. an *ALK* gene rearrangement) to remain true to the selection criteria applied in the trial. This is true of chromogenic in situ hybridisation (CISH) which may be considered an equivalent marker to FISH as it is designed to detect the same biological alteration, (an *ALK* rearrangement) albeit with a different visualisation mechanism, and has been shown to correlate with the results of FISH assays [26].

As ALK (FISH) is the only marker included in a prospective clinical trial for crizotinib therapy, other assays that show changes in *ALK* are in fact **surrogate predictive markers**. If one compares the IHC markers to the *ALK* (FISH) assay, it is interesting to note the biological premise for these tests assumes an increase in expression of the protein, or a component of the protein. In the majority of cases, comparison would suggest this is a valid assumption, but this still does not test for the same outcome [26–28]. IHC, on the other hand may detect a potentially treatment relevant increase in ALK protein expression in the absence of a translocation. Nonetheless, it remains to be seen whether such cases occur and what the biological significance is. RT-PCR also detects the presence of the EML4-ALK translocation but is not currently advocated for routine use as it may not identify ALK fusions with rarer fusion partners [29].

A more direct translation from PCR based testing to IHC is seen in the mutation specific antibodies used in detection of mutations in the *BRAF* gene [30]. Markers such as this offer a binary interpretation as staining should be lacking in the absence of the mutation. These antibodies offer the pathologist an opportunity to view the mutated cells in a morpho-molecular context. In contrast to using IHC as a substitute for FISH in *ALK* translocation detection, mutation specific

antibodies do not require an assumption of biological activity and are thus more directly applicable to clinical use. However, in lung cancer the range of mutations characterised for *EGFR* make the implementation of IHC based mutation screens unfeasible and the use of mutation specific IHC is not encouraged for prediction of response to gefitinib/erlotinib [29]. Interestingly, the CAP/IASLC/AMP guidelines do view the use of ALK (IHC) as acceptable if the test is appropriately validated [29]. Ideally validation would be carried out to the molecular assay validation standards published by the CAP [31].

Another source of uncertainty in using surrogate IHC markers arises from the subjectivity inherent in pathological scoring of IHC stained slides. The marker Ki67 has been suggested to be of prognostic significance in breast cancer [32], however, the inter-laboratory comparability of Ki67 scoring has been difficult to standardise [33]. If IHC is to become a front-line molecular marker, efforts must be made to ensure that standardisation and comparability are designed into systems for evaluating staining of these markers. While it is desirable to think that image analysis software may permit this level of standardisation, this is not an established approach in many laboratories.

In selecting a surrogate marker over a standard-of-care or clinical trial marker, we should ensure that the same standards of method validation and an appropriate number of cases per annum are analysed. Adherence to good practice aside, it is imperative that one recognises that the biological effect may differ across analytical platforms and that deviation from the clinical-trial detection technology may result in spurious inclusion or exclusion of patients for therapy. For this reason it is desirable to use the clinical trial analytical target

where possible until the validity of an alternative target has been established and replicated in the literature.

**Predictive versus prognostic biomarkers**

A popular definition for a prognostic biomarker is one that "provides information about the patients overall cancer outcome, regardless of therapy" while a predictive biomarker is one that "gives information about the effect of a therapeutic intervention" [34]. Some markers are both prognostic and predictive or are a combination of prognostic and predictive as illustrated in Figure 1. A prime example of a marker that is both prognostic and predictive is the molecule BRAF, which when mutated in colorectal cancer (CRC) can be predictive of response to anti-EGFR therapies [35] but is also a negative prognostic marker in the same condition [36]. While the predictive role of *BRAF* mutation in colorectal cancer still remains controversial, it highlights a source of potential confusion regarding the distinction between prognostic and predictive markers. Commentaries on this vocabulary are notable in the literature [37] and highlight that nomenclature and study design both contribute to uncertainty regarding the merits of performing a given molecular assay.

In lung cancer, *EGFR* mutation is predictive of response to EGFR-TKIs such as afatinib [38], gefitinib [8] or erlotinib [39]. Researchers have provided evidence for a prognostic role for *EGFR* mutation and demonstrated that stage I-III lung cancer patients with an *EGFR* mutation have a lower risk of death post resection than those without [40]. This issue is not without conflicting views, as a subsequent study found that *EGFR* mutation was predictive of response to EGFR-TKIs but not prognostic [41]. The authors of this study demonstrated that the

association of *EGFR* mutation with other good prognostic factors such as smoking status may confound some analyses. Both authors recognised that *EGFR* mutation may confer increased survival via EGFR-TKI therapy and D'Angelo *et al* did recognise this as a potential confounding factor in their analyses [40].

While the debate over the utility of which markers can be used as predictive and which can be used for their prognostic value continues, the important thing to keep in mind, that these markers need to be used appropriately. Understanding the difference between prognostic and predictive and knowing that there can be overlap is the first step in defining a marker's clinical utility. This base knowledge can then be built upon with scientific effort to define marker specific roles in predicting the natural course of disease and how a disease will respond to therapy.

**Sampling and molecular heterogeneity**

While intratumoral heterogeneity was obvious at an immunohistochemical level since at least the 1980's [42], the source of this heterogeneity at a genetic level had yet to be characterized. The fact that cytogenetic analysis became the first mainstream genetic clinical technique allowed the early identification of genetic heterogeneity at the chromosomal level [43]. The subsequent development of PCR as a biological research technique permitted the analysis of the heterogeneity of single gene defects within a single tumour [44].

More recently, the advent of high throughput sequencing has permitted the analysis of both intratumoral and metastatic tumour heterogeneity to reveal patterns of branched evolution. The study by Gerlinger *et al* [45] highlighted the degree to which tumour mutation status can vary within an individual and

across numerous genes in renal carcinoma. Single gene studies have demonstrated *PIK3CA* mutational discordance between primary and asynchronous metastatic lesions and additionally revealed intratumoral *PIK3CA* mutational heterogeneity in breast cancer [46]. Work in metastatic colorectal cancer has demonstrated that metastatic lesions are more likely to have concordant *RAS* (*KRAS* or *NRAS*) mutations, less frequent *BRAF* mutations and more frequent *TP53* mutations than the matched primary lesion [47]. The decrease in *BRAF* mutation positivity from primary CRC lesions to their associated metastases was confirmed in a separate study [48]. A study examining intratumoral heterogeneity in primary advanced CRC found discordance in 7% of cases for *KRAS* mutation testing leading the authors to suggest that combining DNA from more than one sample is advisable [49]. For *BRAF* mutations in Metastatic Melanoma (MM), two studies found a moderate level of discordance between primary and metastatic lesions, however, the discrepancy was noted as an absence of the *BRAF* mutation in either the primary or metastatic lesions for both reports [50,51].

For lung cancer, it is established that complete concordance for the canonical *EGFR* mutation is not a biological reality. One study by Park *et al* identified that nearly 12% of paired lesions were found to have a discrepant EGFR mutation result [52]. Interestingly, Park *et al* reported that for the 12 discordant results in their study, 11 of these were due to the absence of a mutation in the metastatic lesion. Whether this represents a biological phenomenon or a technical issue with testing of metastatic lesions remains to be fully established. A next generation sequencing (NGS) study examining both driver and passenger mutations in non-small-cell lung cancer found that concordance for frequently

occurring mutations was 94%, whereas likely passenger alterations showed a lower concordance of 63% [53]. Intra-tumoral heterogeneity was also reported by a study focussing on *EGFR* mutations and correlating these responses to gefitinib response [54].

In contrast to the moderate inter- and intra-tumour discordance noted in the previous studies, a uniform distribution of mutations intra-tumourally was reported for EGFR mutations by Yatabe *et al* [55]. The authors proffered a mechanism by which intra-tumour EGFR copy number variation give rise to pseudo-heterogeneity by varying the mutant allelic frequency within the tumour. In their discussion the authors of the study point to the work of Soh *et al* who found evidence for a phenomenon termed Mutant Allele Specific Imbalance (MASI), which leads to an increased copy number for mutant alleles versus wild type alleles. When coupled with the knowledge that *EGFR* amplification occurs during progression and invasion [56], the authors suggested that this manifests an artificial level of tumour mutational heterogeneity which may not have been controlled for in previously published studies.

As the technical and biological basis for mutational heterogeneity has yet to be fully elucidated, deciding whether to err on the side of caution or practicality can require a great deal of forethought. The authors of the most current guidelines on *EGFR* and *ALK* mutation testing in NSCLC have suggested that the evidence would support the testing of either metastatic or primary lesions [29]. As further studies are completed with more sensitive multi-gene assays, one might expect that the uncertainty surrounding sampling of tumours will decrease.

**Population differences in mutation prevalence can affect assay predictive values**

When selecting an assay for clinical use, one must be careful to select those techniques that deliver a level of performance acceptable for clinical use. For predictive markers, one must ensure that the positive and negative predictive values are appropriate. For example, a high positive predictive value (PPV) is necessary in cases where a false positive results in prescription of a therapy that shortens rather than prolongs a patient's life. This is the case for gefitinib, where those with an *EGFR* wild-type tumour have a shorter progression free survival than those with an *EGFR* mutation [8]. The level of confidence one would like to have in an assay is a clinical decision and should be based on the potential ill-effects that may arise from an incorrect result being issued.

One should remain cognisant of the fact that predictive values are calculated using a prior probability estimate. In the case of molecular markers one might use published figures for mutation rates in particular tumours. At a practical level this means that changes in overall mutation rates may affect the positive or negative predictive value of an assay. These changes may result from ethnic, social, clinical or demographic changes in a population. As a general rule for a high PPV one requires a combination of an assay with a high specificity for, and a high prevalence of, the mutation being tested in the target population. If one examines the figures for *EGFR* mutation rates from the most recent international guidelines [12], it is clear that the PPV of the assay might vary depending on the population being tested. As an example, the mutation rate is three fold higher in Asian/Indian populations than Hispanic populations (52% vs 17% respectively). If we add to this the fact that females have approximately twice the frequency of

EGFR mutations, then we are faced with a prevalence of 78% in Asian/Indian females versus 8.5% in Hispanic males. Clinically, an assay that is optimised for an Asian/Indian female population may not be directly transferrable to a Hispanic male population. While this is an extreme example, it underscores the importance of calculating the clinical performance characteristics of an assay prior to implementation.

The uncertainty inherent in molecular testing of prognostic and predictive markers for different populations or demographic groups may be specific to particular circumstances and may only be an issue for a subset of molecular tests. Fortunately, calculation of the assay predictive values can assist in controlling for these variations and this calculation should be implemented in routine practice.

**Implications of multi-gene testing for future diagnostic platforms.**

The release of data from The Cancer Genome Atlas (TCGA) for squamous cell carcinoma of the lung [57] and the future release of data for lung adenocarcinoma are likely to lead to an increase in the range of mutations being examined in clinical trials. Currently, a number of institutions provide multiple gene hotspot testing on a research basis. However, more formal prospective trials are being implemented to evaluate the use of this form of testing in a clinical setting. The SHIVA trial in Europe [58] aims to profile patients' tumours based upon the detection hotspot mutations in known and putative treatment linked genes.

The majority of multigene testing panels are underpinned by NGS which permits the analysis of Megabases to Gigabases of genetic information in a single run by

sequencing huge numbers of sequences in parallel [59]. Performed singly, it might take a single researcher weeks or even months to generate mutation results for a 50 gene panel for eight patients using Sanger sequencing. NGS is critical to the current trend towards multi-gene testing as, depending on the platform, the same analysis and reporting can be completed in days.

While the suitability of NGS for clinical use requires many technical considerations [59–62], when one moves beyond the technical and into the clinical implementation of NGS, even more questions need to be answered. To begin, for each additional assay that is added to a testing panel, the probability of a false positive result increases. For example, if we assume that all assays in a 50 gene NGS panel have a specificity of 99.5% and each result is independent of the next, then we would see a false positive every 200 assays. Therefore, for wild-type tumours with this 50 gene panel and this level of specificity, we may expect one false positive for every four patients tested. It is then necessary to find mechanisms to control for this false positive rate, reduce the number of target genes or invest in increasing the assay specificity. In addition to the risks inherent in multiple testing, one is faced with the problem of which mutations to report and how to report them. Not only does the laboratory need to implement the computational infrastructure to generate human-readable reports [60], but the reports must ideally comply with best practice guidelines that are currently in place for reporting of molecular test results [63].

One is also faced with the dilemma of how to report novel mutations in known therapy guiding genes as well as novel mutations in putative therapy guiding genes. As an example, a body of evidence exists to support the prescription of gefitinib or erlotinib in NSCLC when a patient exhibits a known mutation in

*EGFR*. If a laboratory detects a novel *EGFR* mutation, it is faced with the dilemma of how to report this finding. The oncologist is also faced with an equally perplexing dilemma of whether or not to treat with the established targeted therapy based on this finding. If a laboratory elects to report only known mutations, it may store the remaining data for potential mining at a later stage if a new therapy arises. However, in this scenario, the laboratory will also need to consider whether it will inform previously tested patients if they become candidates for this novel therapy based on a previous positive test for the mutation of interest. The ethical issues involved with testing such a broad range of genes have yet to play out in routine diagnostics, However, it would be prudent for oncologists and pathologists to engage at a local level if NGS is being implemented, to ensure that potential ethical problems are discussed and clarified before testing commences.

While expansion of the number of mutations currently being tested seems to be inevitable, it is clear that the medium term view places a dependence on NGS to help laboratories carry out this testing. However, the novelty of NGS makes it a bleeding-edge technology, so the laboratory that implements it for clinical diagnostics must be ready to invest in the local expertise and consultation to support the platform.

**Regulation of Companion Diagnostics**

Within the United States both drugs and medical devices fall within the remit of the Food and Drug Administration (FDA) [64]. Recognising the pivotal importance of the companion diagnostic assay to the correct administration of a select group of therapies, the FDA issued a draft guideline in 2011 addressing the

issue of companion diagnostics. In this, the FDA encouraged the co-development of companion diagnostics for therapeutic products that depend on the use of a diagnostic assay to ensure appropriate administration of therapy [65]. They also defined companion diagnostics as "an in vitro diagnostic device that provides information that is essential for the safe and effective use of a corresponding therapeutic product".

While the legislation surrounding the licensing of companion diagnostics is beyond the scope of this article, it is worth exploring the drawbacks of having to approve a diagnostic assay for use with a particular therapy. As of November 2013 the FDA's page on companion diagnostics has nineteen companion diagnostic assays listed, of which ten are companion diagnostics assays for either trastuzumab or pertuzumab [66]. The remaining nine assays, outlined in Figure 2 represent those assays for which a single companion diagnostic is approved and in use in routine practice. From the diagram it is evident that each of the nine therapies is linked to a single companion diagnostic assay. From a laboratory perspective this makes testing quite problematic as each laboratory would have to run a separate test for each therapy under consideration. For example, if afatinib is being considered, the laboratory will need to assess the patient's EGFR mutation status using the QIAGEN Therascreen assay, whereas the Roche Cobas assay will need to be used to detect the same mutations if erlotinib is being considered. As it is conceivable to think that both may be under consideration simultaneously, it would be incumbent on the laboratory to have both platforms available for use. This also means that laboratories with a strong track record and expertise in one molecular technology would be prohibited

from using that technology to create a laboratory developed test that may be analytically and clinically superior to the approved companion diagnostic.

While the debate surrounding the FDA approval of assays is likely to continue, it is important to remain aware that this linked approval of therapy and companion diagnostic does not apply in Europe. Erlotinib, for example, is indicated by the European Medicines Agency (EMA) for the "first-line treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) with EGFR activating mutations" [67]. In their report, the EMA make no recommendation regarding the assay to be used for *EGFR* mutation detection save for specifying that the methodology is well validated and robust. As an increasing number of therapies that depend on companion diagnostics are released to the market, it is likely that the advantages and disadvantages of both the FDA and EMA approval mechanisms will become evident.

## Conclusions

Within the field of molecular diagnostics for oncology there exist areas of confusion or uncertainty. A major contributor to this uncertainty is the novelty of both the mutations being investigated and the application of molecular techniques to an existing area of pathology. However, by clearly defining the problems one can be forearmed with the tools to circumvent or eliminate them. Thus, this article has explored key areas that are sources of confusion or misinterpretation in molecular testing with a particular focus on predictive and prognostic biomarkers.

As we move forward as a scientific community and seek to bring the world of molecular diagnostics into practical clinical practice, there are a few key aspects to remember. To begin, one must first consider the appropriateness of a biomarker for clinical use i.e. how would the results of this assay alter the course of patient's therapy. Understanding the difference between prognostic and predictive and knowing that there can be overlap is the first step in defining a marker's clinical utility and purpose. Most importantly, the chosen marker should be supported by research sufficient to give confidence in the marker's ability to deliver a clinically meaningful result.

When one moves into the realm of the laboratory there are numerous considerations which affect the practicality of testing. One must be guarded when choosing between those assays that mimic the conditions of a clinical trial and those that measure or detect surrogates of the clinical trial marker; ALK(IHC) as a predictive marker for crozotinib therapy would be an example of the latter. It is imperative that one recognises that the biological effect may be different across analytical platforms and that deviation from the clinical-trial detection technology may result in spurious inclusion or exclusion of patients for therapy. For this reason it is desirable to use the clinical trial analytical target where possible until the validity of an alternative target has been established and replicated in the literature.

Each assay will need to be optimised for the target population and analytical substrate; intra- and inter-tumoural heterogeneity will affect which samples are suitable for analysis and fluctuations in mutation rates due to geographic, ethnic and clinical may adversely skew assay predictive values so may need to be considered before assay implementation. Additionally, while many authors

recognise the potential for NGS as a diagnostic technology, it is in its infancy for clinical diagnostics and implementation requires consideration of clinical, ethical and technical issues before local acceptance for clinical use.

Lastly, one must always consider the regulatory component when designing or validating a diagnostic test. It is clear that the debate surrounding the FDA approval of assays is likely to continue, but the growing trend towards NGS makes this approach seem untenable. The differing approaches in Europe and the USA regarding licencing of companion diagnostics may permit a sensible comparison between both approaches in the near future.

## Conflict of Interest Statement

None Declared

## References

[1] Scagliotti GV, Parikh P, von Pawel J, Biesma B, Vansteenkiste J, Manegold C, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. J Clin Oncol 2008;26:3543–51.

[2] Robert N, Leyland-Jones B, Asmar L, Belt R, Ilegbodu D, Loesch D, et al. Randomized phase III study of trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 2006;24:2786–92.

[3] Cohen MH, Williams G, Johnson JR, Duan J, Gobburu J, Rahman A, et al. Approval Summary for Imatinib Mesylate Capsules in the Treatment of Chronic Myelogenous Leukemia. Clin Cancer Res 2002;8:935–42.

[4] Giorgi U De, Verweij J, Giorgi U De, Verweij J. Imatinib and gastrointestinal stromal tumors : Where do we go from here ? Minireview Imatinib and gastrointestinal stromal tumors : Where do we go from here ? 2005:495–501.

[5] Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med 2011;135:537–43.

[6] Chung J-Y, Braunschweig T, Williams R, Guerrero N, Hoffmann KM, Kwon M, et al. Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. J Histochem Cytochem 2008;56:1033–42.

[7] 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.

[8] Mok TS, Wu Y, Thongprasert S, Yang C-H, Chu D-T, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947–57.

[9] Pirker R, Herth FJF, Kerr KM, Filipits M, Taron M, Gandara D, et al. Consensus for EGFR mutation testing in non-small cell lung cancer: results from a European workshop. J Thorac Oncol 2010;5:1706–13.

[10] Dillon B, Naidoo B, Knight H, Clark P. NICE guidance on erlotinib for first-line treatment of EGFR-TK mutation-positive advanced or metastatic non-small-cell lung cancer. Lancet Oncol 2012;13:764–5.

[11] National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology, Non-Small Cell Lung Cancer Version 1.2014 2013.

[12] Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, et al. Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors: Guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Patho. Arch Pathol Lab Med 2013.

[13] Lord RVN, Brabender J, Gandara D, Alberola V, Camps C, Domine M, et al. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. Clin Cancer Res 2002;8:2286–91.

[14] Simon GR, Sharma S, Cantor A, Smith P, Bepler G. ERCC1 expression is a predictor of survival in resected patients with non-small cell lung cancer. Chest 2005;127:978–83.

[15] Olaussen KA, Dunant A, Fouret P, Brambilla E, André F, Haddad V, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med 2006;355:983–91.

[16] Mascaux C, Iannino N, Martin B, Paesmans M, Berghmans T, Dusart M, et al. The role of RAS oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. Br J Cancer 2005;92:131–9.

[17] Slebos RJ, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJ, et al. K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med 1990;323:561–5.

[18] Tsao M-S, Aviel-Ronen S, Ding K, Lau D, Liu N, Sakurada A, et al. Prognostic and predictive importance of p53 and RAS for adjuvant chemotherapy in non small-cell lung cancer. J Clin Oncol 2007;25:5240–7.

[19] Subramanian J, Simon R. Gene expression-based prognostic signatures in lung cancer: ready for clinical use? J Natl Cancer Inst 2010;102:464–74.

[20] Zhu C-Q, Shih W, Ling C-H, Tsao M-S. Immunohistochemical markers of prognosis in non-small cell lung cancer: a review and proposal for a multiphase approach to marker evaluation. J Clin Pathol 2006;59:790–800.

[21] Le Tourneau C, Kamal M, Trédan O, Delord J-P, Campone M, Goncalves A, et al. Designs and challenges for personalized medicine studies in oncology: focus on the SHIVA trial. Target Oncol 2012;7:253–65.

[22] 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 2013:130601060020006.

[23] Kwak EL, Bang Y-J, 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.

[24] Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res 2009;15:3143–9.

[25] Togashi Y, Soda M, Sakata S, Sugawara E, Hatano S, Asaka R, et al. KLC1-ALK: a novel fusion in lung cancer identified using a formalin-fixed paraffin-embedded tissue only. PLoS One 2012;7:e31323.

[26] Kim H, Yoo S-B, Choe J-Y, Paik JH, Xu X, Nitta H, et al. Detection of ALK gene rearrangement in non-small cell lung cancer: a comparison of fluorescence in situ hybridization and chromogenic in situ hybridization with correlation of ALK protein expression. J Thorac Oncol 2011;6:1359–66.

[27] Yi ES, Boland JM, Maleszewski JJ, Roden AC, Oliveira AM, Aubry M-C, et al. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell

lung carcinoma: IHC score algorithm for FISH. J Thorac Oncol 2011;6:459–65.

[28] Paik JH, Choe G, Kim H, Choe J-Y, Lee HJ, Lee C-T, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer: correlation with fluorescence in situ hybridization. J Thorac Oncol 2011;6:466–72.

[29] Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, et al. Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors: Guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Patho. J Thorac Oncol 2013;XXX:1–37.

[30] Long G V, Wilmott JS, Capper D, Preusser M, Zhang YE, Thompson JF, et al. Immunohistochemistry Is Highly Sensitive and Specific for the Detection of V600E BRAF Mutation in Melanoma. Am J Surg Pathol 2012.

[31] Jennings L, Van Deerlin VM, Gulley ML. Recommended principles and practices for validating clinical molecular pathology tests. Arch Pathol Lab Med 2009;133:743–55.

[32] Urruticoechea A, Smith IE, Dowsett M. Proliferation marker Ki-67 in early breast cancer. J Clin Oncol 2005;23:7212–20.

[33] Polley M-YC, Leung SCY, McShane LM, Gao D, Hugh JC, Mastropasqua MG, et al. An International Ki67 Reproducibility Study. J Natl Cancer Inst 2013.

[34] Oldenhuis CN a M, Oosting SF, Gietema J a, de Vries EGE. Prognostic versus predictive value of biomarkers in oncology. Eur J Cancer 2008;44:946–53.

[35] Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 2008;26:5705–12.

[36] Safaee Ardekani G, Jafarnejad SM, Tan L, Saeedi A, Li G. The prognostic value of BRAF mutation in colorectal cancer and melanoma: a systematic review and meta-analysis. PLoS One 2012;7:e47054.

[37] Italiano A. Prognostic or predictive? It's time to get back to definitions! J Clin Oncol 2011;29:4718; author reply 4718–9.

[38] Sequist L V, Yang JC-H, Yamamoto N, O'Byrne K, Hirsh V, Mok T, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. J Clin Oncol 2013;31:3327–34.

[39] Cadranel J, Mauguen A, Faller M, Zalcman G, Buisine M-P, Westeel V, et al. Impact of systematic EGFR and KRAS mutation evaluation on progression-free survival and overall survival in patients with advanced non-small-cell lung cancer treated by erlotinib in a French prospective cohort (ERMETIC project--part 2). J Thorac Oncol 2012;7:1490–502.

[40] D'Angelo SP, Janjigian YY, Ahye N, Riely GJ, Chaft JE, Sima CS, et al. Distinct clinical course of EGFR-mutant resected lung cancers: results of testing of 1118 surgical specimens and effects of adjuvant gefitinib and erlotinib. J Thorac Oncol 2012;7:1815–22.

[41] Kim YT, Seong YW, Jung YJ, Jeon YK, Park IK, Kang CH, et al. The presence of mutations in epidermal growth factor receptor gene is not a prognostic factor for long-term outcome after surgical resection of non-small-cell lung cancer. J Thorac Oncol 2013;8:171–8.

[42] Enblad P, Glimelius B, Busch C, Pontén J, Påhlman L. Antigenic heterogeneity and individuality in adenocarcinomas of the rectum and their secondaries. Br J Cancer 1987;55:503–8.

[43] Nordenson I, Ljungberg B, Roos G. Chromosomes in renal carcinoma with reference to intratumor heterogeneity. Cancer Genet Cytogenet 1988;32:35–41.

[44] Lönn U, Lönn S, Nilsson B, Stenkvist B. Intratumoral heterogeneity for amplified genes in human breast carcinoma. Int J Cancer 1994;58:40–5.

[45] Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.

[46] Dupont Jensen J, Laenkholm A-V, Knoop A, Ewertz M, Bandaru R, Liu W, et al. PIK3CA mutations may be discordant between primary and corresponding metastatic disease in breast cancer. Clin Cancer Res 2011;17:667–77.

[47] Vakiani E, Janakiraman M, Shen R, Sinha R, Zeng Z, Shia J, et al. Comparative genomic analysis of primary versus metastatic colorectal carcinomas. J Clin Oncol 2012;30:2956–62.

[48] Santini D, Spoto C, Loupakis F, Vincenzi B, Silvestris N, Cremolini C, et al. High concordance of BRAF status between primary colorectal tumours and related metastatic sites: implications for clinical practice. Ann Oncol 2010;21:1565.

[49] Richman SD, Chambers P, Seymour MT, Daly C, Grant S, Hemmings G, et al. Intra-tumoral heterogeneity of KRAS and BRAF mutation status in patients with advanced colorectal cancer (aCRC) and cost-effectiveness of multiple sample testing. Anal Cell Pathol (Amst) 2011;34:61–6.

[50] Boursault L, Haddad V, Vergier B, Cappellen D, Verdon S, Bellocq J-P, et al. Tumor homogeneity between primary and metastatic sites for BRAF status in metastatic melanoma determined by immunohistochemical and molecular testing. PLoS One 2013;8:e70826.

[51] Colombino M, Capone M, Lissia A, Cossu A, Rubino C, De Giorgi V, et al. BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. J Clin Oncol 2012;30:2522–9.

[52] Park S, Holmes-Tisch AJ, Cho EY, Shim YM, Kim J, Kim HS, et al. Discordance of molecular biomarkers associated with epidermal growth factor receptor pathway between primary tumors and lymph node metastasis in non-small cell lung cancer. J Thorac Oncol 2009;4:809–15.

[53] Vignot S, Frampton GM, Soria J-C, Yelensky R, Commo F, Brambilla C, et al. Next-generation sequencing reveals high concordance of recurrent somatic alterations between primary tumor and metastases from patients with non-small-cell lung cancer. J Clin Oncol 2013;31:2167–72.

[54] Taniguchi K, Okami J, Kodama K, Higashiyama M, Kato K. Intratumor heterogeneity of epidermal growth factor receptor mutations in lung cancer and its correlation to the response to gefitinib. Cancer Sci 2008;99:929–35.

[55] Yatabe Y, Matsuo K, Mitsudomi T. Heterogeneous distribution of EGFR mutations is extremely rare in lung adenocarcinoma. J Clin Oncol 2011;29:2972–7.

[56] Sholl LM, Yeap BY, Iafrate a J, Holmes-Tisch AJ, Chou Y-P, Wu M-T, et al. Lung adenocarcinoma with EGFR amplification has distinct clinicopathologic and molecular features in never-smokers. Cancer Res 2009;69:8341–8.

[57] Cancer T, Atlas G. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489:519–25.

[58] A Randomized Phase II Trial Comparing Therapy Based on Tumor Molecular Profiling Versus Conventional Therapy in Patients With Refractory Cancer n.d.

[59] Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet 2010;11:31–46.

[60] Nekrutenko A, Taylor J. Next-generation sequencing data interpretation: enhancing reproducibility and accessibility. Nat Rev Genet 2012;13:667–72.

[61] Gargis AS, Kalman L, Berry MW, Bick DP, Dimmock DP, Hambuch T, et al. Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol 2012;30:1033–6.

[62] Gullapalli RR, Desai K V, Santana-Santos L, Kant J a, Becich MJ. Next generation sequencing in clinical medicine: Challenges and lessons for pathology and biomedical informatics. J Pathol Inform 2012;3:40.

[63] Gulley ML, Braziel RM, Halling KC, Hsi ED, Kant J a, Nikiforova MN, et al. Clinical laboratory reports in molecular pathology. Arch Pathol Lab Med 2007;131:852–63.

[64] US Food and Drug Administration. FDA Basics - What does FDA regulate? n.d.

[65] US Food and Drug Administration. Guidance Documents (Medical Devices and Radiation-Emitting Products) - Guidance for Industry and Food and Drug Administration Staff - In Vitro Companion Diagnostic Devices n.d.

[66] US Food and Drug Administration. In Vitro Diagnostics - Companion Diagnostic Devices: In vitro and Imaging Tools n.d.

[67] European Medicines Agency. Tarceva: EPAR - Product Information. Annex I. n.d.

**Figure 1: Simulated trial data for prognostic and predictive biomarkers**

Simulated trial data and the associated Kaplan-Meier plots were generated to illustrate examples of how a biomarkers may stratify patient populations based upon a marker being predictive, prognostic, neither or both. Patients were assumed to be given either an interventional therapy or a control and tested for the presence of a biomarker.

**Figure 2: Companion diagnostic assays approved by the FDA**

As per the figure legend, assays which are approved for use as companion diagnostics are highlighted in green. The Dako PharmDx antibodies were approved for use when the EGFR expression by IHC was used to determine a candidates eligibility for cetuximab or panitumumab therapy, this is not currently standard of care [11] but remains an approved companion diagnostic. The Roche Cobas and bioMériux THxID differ in that they are listed to detect the V600E or V600E and V600K mutations respectively.

Figure1

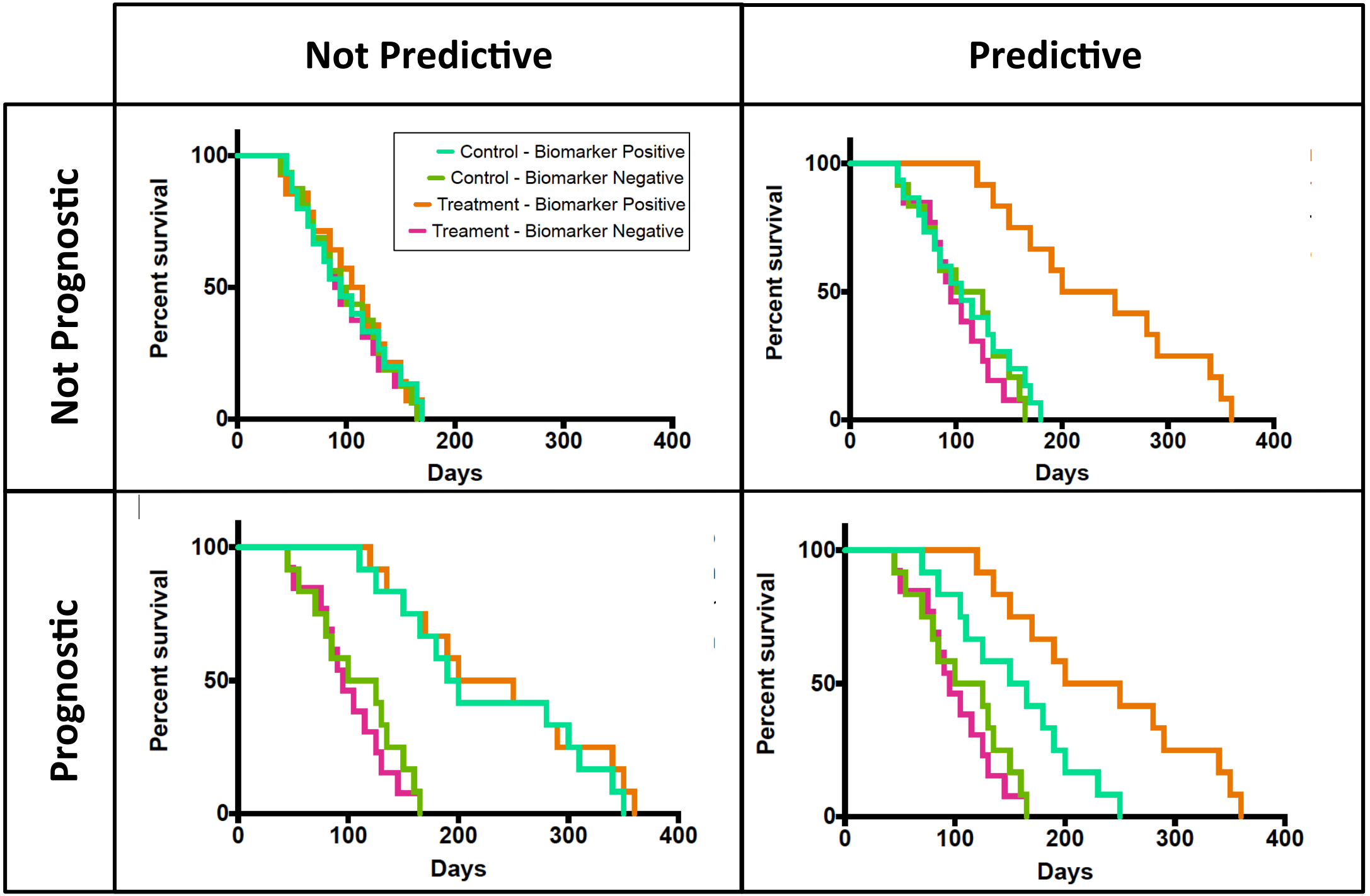
Not Predictive
Predictive
Not Prognostic
Prognostic
Control - Biomarker Positive
Control - Biomarker Negative
Treatment - Biomarker Positive
Treament - Biomarker Negative
Percent survival
Days
0
50
100
0
100
200
300
400

Figure2