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Challenges in molecular testing in non-small cell lung cancer patients with advanced disease

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Summary:

Lung cancer diagnostics have evolved significantly in the previous decade. The challenge of molecular testing to identify an increasing number of potentially clinically actionable genetic variants, using smaller samples obtained via minimally invasive techniques, is significant. Tumour heterogeneity and cancer evolution in response to therapy means that repeat biopsies or circulating biomarkers are likely to be increasingly useful to adapt treatment as resistance develops. We highlight some of the current challenges faced in clinical practice for molecular testing of EGFR, ALK and new biomarkers such as PDL1. Implementation of next generation sequencing (NGS) platforms for molecular diagnostics in non-small cell lung cancer is increasingly common allowing testing of multiple genetic variants from a single sample. The use of NGS to recruit to molecularly stratified clinical trials is discussed in the context of the UK Stratified Medicine Programme and The UK National Lung Matrix Trial.

Historical overview of lung cancer diagnostics

Lung cancer is the most common cause of mortality in the UK, accounting for 1 in 5 of all cancer deaths.[1] With the estimated global incidence in 2012 of 1.83 million cases it is important to reflect that a century ago, lung cancer diagnosis was a rare event. In comparison to the current epidemic, in 1912 Isaac Adler's collection of 374 case reports in his publication *Primary Malignant Growths of the Lungs and Bronchi* represented the entire known global incidence at the time. A century later the WHO histological classification of malignant epithelial tumours of the lung recognizes different histologies with many variants for each subtype and analyses from next generation sequencing (NGS) studies have divided this disease into molecular subtypes defined by distinct somatic alterations.[2-4] This review will focus on key challenges faced in current clinical practice for molecular testing in non-small cell lung cancer (NSCLC). In broad terms the challenges are technical, logistical and related to tumour biology and some of the pertinent issues are highlighted (Figure 1).

Identification of tumour histology

Historically the treatment focus for those with advanced NSCLC was selection of an appropriate cytotoxic chemotherapy regimen irrespective of histological subtype. Several large studies were published that showed the efficacy of various platinum doublet combinations were comparable but with differing drug specific toxicities.[5-7] However accurate classification of NSCLC subtype has become fundamental in the management of advanced NSCLC following the results of phase III clinical trials showing improved progression free survival in EGFR mutation positive adenocarcinoma treated with EGFR tyrosine kinase inhibitors (TKI),[8,9] and improved overall survival with pemetrexed in the first line and maintenance setting for those with non-squamous histology.[10][11]

[12,13]The number of tumours that cannot be given an accurate histological diagnosis (i.e. adenocarcinoma versus squamous cell carcinoma) has reduced significantly with the use of immunohistochemical markers. The use of markers for p63, p40 and cytokeratin CK 5/6 help to identify squamous cell carcinomas, while thyroid transcription factor 1 (TTF1), Napsin A and CK7, as well as mucin stains, are indicative of adenocarcinomas.[14] However interpretation of immunohistochemistry panels still requires the expertise of an experienced histopathologist, as markers are not reliable in isolation.[15] TTF1 for example, a marker

synonymous with adenocarcinoma, is expressed in only 80-90% of cases and is commonly expressed in neuroendocrine tumours.[14,16] Immunohistochemistry can only be meaningfully interpreted in a detailed morphological context.

Sampling challenges in advanced NSCLC

The analysis of lung cancer tissue is particularly challenging as primary lung tumours often show much lower tumour cellularity than other tumour types. Even with macroscopic selection of areas of frank carcinoma the tumour purity (the fraction of a given region containing tumour cells) can often be <20% because of the high proportion of stromal cells, lymphocytic infiltration and necrosis (unpublished observations from the UK Lung TRACERx longitudinal cohort study).[17].

This challenge is compounded by the nature of the specimen types routinely received by histopathology and molecular diagnostics laboratories. Presentation with metastatic disease is common and only a small proportion of patients with NSCLC undergo curative surgical resection.[18] The large tissue samples obtained via open thoracotomy (wedge resection, lobectomy, pneumonectomy) are usually of sufficient quantity and quality for a number of histological and molecular assays if handled appropriately. However patients with advanced disease are predominately diagnosed with CT guided percutaneous or US guided endoscopic biopsy with 18 gauge needles or with fine needle aspiration. These patients are the cohort where molecular diagnostics are most important for determining the standard of care and enabling participation in clinical trials yet the sample quality and quantity from such needle biopsies is the most limiting for histological and molecular testing.

Obtaining adequate tissue for diagnosis, tissue sub-typing, molecular profiling and treatment planning are therefore key to patient management. The target tumour is not always easily accessible in patients presenting with a probable lung cancer. The development of endobronchial ultrasound transbronchial needle aspiration (EBUS-TBNA) is proving increasingly important in the investigation and management of thoracic malignancies as it offers a minimally invasive approach to sampling of mediastinal lymph nodes and masses. EBUS-TBNA is now increasingly embedded in routine clinical practice with wider use beyond high volume tertiary centres in the UK and USA. It is now generally accepted that EBUS-TBNA alone or in combination with endoscopic ultrasound fine needle aspiration (EUS-FNA) can potentially spare surgical mediastinoscopy or thoracotomy in the staging of NSCLC.[19,20]

Importantly, EBUS-TBNA also offers the possibility of combining diagnosis and staging as a single procedure in patients with suspected lung cancer. In contrast to tissue biopsies or surgical samples that allow sub-typing (adenocarcinoma versus squamous cell carcinoma) on morphological criteria alone in the majority of cases, evaluation of cytological specimens obtained by EBUS-TBNA poses additional challenges that can be partly overcome with wider use of immunohistochemistry.[21]

Identifying driver mutations, such as EGFR and ALK, in these small samples is central to management of patients with advanced disease. Whether molecular analysis is successfully performed depends on the absolute number of tumour cells, the proportion of tumour cells compared to total nucleated cells present and the method used for molecular analysis. In case of EBUS-derived samples, there is evidence to conclude that simple mutation analysis (EGFR, KRAS, ALK) can be successfully performed in most cases.[22-24] The use of multi-gene targeted NGS panels, using only nanograms of DNA, to sequence fine needle aspiration samples is achievable and is becoming more commonly used in clinical practice.[25,26] Whole exome sequencing (WES) and whole genome sequencing (WGS) analysis which require greater amounts of DNA, micrograms in the case of WGS, will be more challenging from EBUS-TBNA samples.

Current challenges in molecular diagnostics for EGFR mutation analysis in clinical practice

The initial randomised phase II studies of gefitinib demonstrated clinical activity,[27,28] and phase III studies although negative for the primary outcome measure, suggested a benefit in patients with adenocarcinoma, those of Asian origin and never smokers.[29,30] During this period a number of seminal case series identified EGFR mutations as a marker of sensitivity to EGFR TKIs,[31-33] and analysis of samples from these early trials supported this.[34] Subsequent phase III trials have incorporated EGFR mutation status and showed higher response rates and progression free survival (PFS) in patients with EGFR activating somatic mutations treated with EGFR TKIs compared to when treated with chemotherapy.[8,9,35]

Development of diverse mechanisms of resistance and selection of resistant clones in response to treatment

The common EGFR mutations are located in the tyrosine kinase domain (exon 18-21) of the

139 EGFR gene, with detection of L858R and deletions in exon 19 the clinical priority as these
140 determine sensitivity to first and second generation TKIs.[36] The T790M mutation in exon 20
141 results in resistance to these therapies.[37] Sensitive assays suggest that tumour clones
142 harbouring the T790M mutation are often detectable prior to initiation of a first generation
143 TKI but can also occur by genetic evolution in T790M mutation negative drug tolerant cells in
144 response to treatment.[38-40] Identification of this resistance mutation is more critical
145 following the development of the third generation EGFR TKIs active against T790M mutation
146 positive NSCLCs.[41,42] But whether these T790M resistant clones pre-exist or evolve in
147 response to treatment may have clinical implications with differing sensitivities to third
148 generation TKIs.[40] The capacity for tumours to evolve in response to first generation TKIs
149 results in an additional diverse array of mechanisms of resistance such as amplification of
150 MET, selection for PIK3CA or BRAF mutations and transformation to a small cell
151 phenotype.[43] Clearly, cancer evolution and selection of resistant subclones is not restricted
152 to first generation TKIs. This is highlighted by recent reports of the emergence of T790M
153 mutation negative disease and the development of novel secondary EGFR resistance
154 mutations (C797S) after treatment of T790M mutation positive patients with third
155 generation TKIs.[44,45]
156
157 **EGFR mutation testing**
158 The nature of EGFR sensitizing mutations, being single nucleotide variants (SNV) or short
159 deletions, lends themselves to molecular analysis of formalin fixed small samples which
160 contain fragmented DNA.[46] There are a variety of methods to detect EGFR mutations
161 including conventional Sanger sequencing, amplification refractory mutations systems
162 (ARMS), restriction fragment length polymorphisms and more recently as part of targeted
163 NGS panels.[47,48] Reporting the limitations of an assay along with the result is critical for
164 clinical interpretation. Bi-directional Sanger sequencing without a mutation enrichment step
165 has a lower limit of detection of 10-25% of total DNA meaning that the use of samples with
166 low tumour cellularity can result in false negative mutation calls. Consequently the use of
167 methods that can detect mutations in low tumour cellularity samples (<10%) is
168 recommended. Polymerase chain reaction (PCR) based ultra sensitive and NGS methods can
169 generate artefact mutations leading to false positive results. However techniques, such as
170 duplex sequencing, are being developed to overcome the inherent error rate in sequencing
171 technologis[49] Formalin fixed samples are particularly prone to DNA damage and display
172 disproportionate levels of C>T/G>A changes in the 1-10% allele frequency range which can

173 result in false positive mutation calls.[50] Publication of clinical trials results on response to
174 EGFR TKI in patients with real but less common EGFR mutations can help guide clinical
175 decision-making.[51] Detection of EGFR mutations as part of a WES or WGS analysis allows
176 multiple driver mutations to be queried simultaneously but the performance of
177 bioinformatics tools to call mutations from NGS data varies. Such complexities need to be
178 considered as these technologies are increasingly adopted into mainstream clinical
179 practice.[52-54]
180
181 **EGFR mutations, resistance and tumour heterogeneity**
182 There are very few reports of discordance of EGFR mutation status between primary disease
183 and metastatic sites and these may be due to technical limitations of the assays used.[55] Loss
184 of the EGFR mutation was not a mechanism seen in seminal studies.[43] Studies looking at the
185 extent of intra tumour heterogeneity (ITH) in early lung cancer have shown EGFR to be
186 exclusively a clonal event prevalent throughout the tumour. [56,57] As resistance to EGFR TKI is
187 usually due to acquisition of secondary mutations in EGFR or other driver genes the key
188 challenge at the time of disease progression is to obtain a contemporaneous sample to
189 inform selection of second line therapy. In general the most easily accessible lesion is used
190 but in patients with a poor performance status this may not be a trivial task. Due to tumour
191 heterogeneity it is possible that a single sample may be insufficient to accurately represent
192 all the resistance mechanisms present or the breadth of subclonal driver events present
193 across multiple disease sites following progression on therapy.
194
195 **Current challenges in molecular diagnostics for ALK testing in clinical practice**
196
197 The discovery of an oncogenic anaplastic lymphoma kinase fusion gene (EML4-ALK) in 2007
198 identified another important molecular cohort in NSCLC.[58] Present in 2-7% of NSCLC ALK
199 fusion genes are restricted to adenocarcinoma subtypes and are more common in younger
200 patients and never-smokers.[59-61] Identification of this cohort is critical given the high
201 response rates (57-74%) to ALK inhibition both as a first line therapy and after platinum-
202 based chemotherapy.[59,62,63] Subsequently other rare fusion genes have been identified
203 involving ROS1, with similar exquisite sensitivity to kinase inhibition,[64] but also RET and
204 NTRK where objective response rates are lower.[65,66]
205
206 **ALK fusion gene detection**

Testing for ALK fusion genes brings its own particular set of challenges. ALK is activated by genomic rearrangement, leading to the expression of a chimeric protein containing the effector part of the ALK tyrosine kinase fused to the proximal portion of another protein. In NSCLC cancer this is typically a balanced translocation with the ubiquitously highly expressed EML4 gene,[58] although rarely other partner genes may be involved.[67,68] Expression of the chimeric protein leads to upregulation of mitogenic signalling through the RAS/RAF pathway and interruption of this pathway by ALK inhibitors causes cancer cell death and tumour regression.[62] ALK-mutated tumours often show unusual features on conventional microscopy, such as cribriform growth patterns and 'signet ring' cells with large vacuoles,[69] but this is not sufficiently sensitive or specific to guide treatment.

The first widely adopted test for ALK-driven tumours was FISH (fluorescence in situ hybridisation), approved by the FDA (US Food and Drug Administration) in 2011.[70] FISH is a technically demanding method, requiring specialised equipment and experienced practitioners. Tissue sections or cytology specimens are subjected to a protocol that labels either side of the ALK breakpoint locus with red and green fluorescent DNA probes. In non-transformed cell nuclei the coloured dots overlap and look yellow, while in translocated cells isolated red and/or green signals are seen. For a reliable FISH assay the tissue must be adequate in quantity and quality. This can be more challenging with small biopsy samples which may contain few cells or which show crushing artefacts that can impair interpretation.

In 2015, an immunohistochemistry method was approved by the FDA. This approach is simpler in principle, using an antibody stain to detect abnormal ALK antigen expression. However, currently available antibodies do not give a strong signal and so an additional signal amplification steps needs to be employed. This places the test beyond the capacity of many small labs. Nonetheless, the modified test is cheaper than FISH, easier to interpret, and has the theoretical advantage of additionally detecting ALK expression following rare atypical rearrangements. After much investigation, recent studies suggest immunohistochemistry can be an adequate stand-alone diagnostic, showing extremely high concordance with FISH.[71] UK guidelines do not dictate which test should be applied, and practices vary regionally, though FISH is still often regarded as the 'gold standard' and is considered the definitive test in the US.[48] As our understanding of tumour taxonomy and genotypes advances, it seems inevitable that some form of NGS platform will become the clinical standard for gene fusion detection.[72] These methods have the potential to detect

241 ALK (and other) rearrangements in either a targeted panel or a WES or WGS approach.[73,74]
242 [72]
243
244 **ALK fusion genes, resistance and tumour heterogeneity**
245 ALK fusion genes are considered to be clonal events with minimal discordance between
246 primary and metastatic lesions.[75] They were considered to be mutually exclusive with EGFR
247 mutations however recent reports suggests a small minority of tumours can contain both
248 ALK and EGFR positive clones.[76-78] The mechanisms of resistance seen following ALK
249 inhibitor therapy again demonstrate tumour evolution with secondary mutations in ALK, ALK
250 copy number gain, secondary driver mutations in other genes and outgrowth of ALK fusion
251 gene negative clones reported.[79-82] Consequently contemporaneous sampling of
252 progressive disease, by needle biopsy or analysis of cfDNA, may allow real time analysis of
253 tumour evolution and guide therapy.
254
255 **Integration of multi-gene NGS testing in clinical practice**
256 Routine molecular profiling can be performed at scale on a national level. Large cooperative
257 efforts in France and the USA used combinations of mutation specific PCRs, Sanger
258 sequencing and FISH analysis to assay 6-10 oncogenic drivers in thousands of patients with
259 NSCLC and survival was improved for those treated with gene directed targeted
260 therapies.[83,84] The use of next generation sequencing to simultaneously assay multiple
261 oncogenic drivers is attractive because less DNA is required compared to multiple individual
262 assays, there is a reduction in hands-on laboratory time, and complex FISH analysis for
263 detection of fusion genes may be avoided. A recent NGS approach used an amplicon based
264 approach to assay 14 genes used only 50ng of DNA from FFPE samples.[85] This study
265 provided a comprehensive assessment of the spectrum of mutations, and co-occurrence of
266 mutations, in adenocarcinoma and squamous cell carcinomas with detection turn around
267 times of less than two weeks. These studies and those of The Cancer Genome Atlas highlight
268 the inter-patient molecular heterogeneity of NSCLC (Figure 2). Even within these molecular
269 cohorts intra-tumour heterogeneity could have significant effects on outcome as
270 exemplified by a recent study showing that the clonality of FGFR amplification is an
271 important predictor of response to FGFR inhibition.[86] A deeper understanding of the clonal
272 or subclonal nature of driver events in NSCLC from sufficiently powered studies, is still
273 awaited. Recruitment of patients with rare mutations to molecularly stratified trials is
274 challenging,[87] and some advocate that modifications to existing paradigms in drug

275 development are required in the era of genomic studies and precision medicine.[88] Multi-
276 gene or WES NGS assays are likely to become standard practice in the years to come and the
277 ultimately automated provision of readable, applicable reports of complex genomic data is
278 another important challenge.
279
280 **Current challenges in molecular diagnostics for PDL1 testing in clinical practice**
281
282 Activation of inhibitory T cell checkpoint interactions in established tumours has been
283 demonstrated in a number of solid tumours, including NSCLC, and this suppresses the anti-
284 tumour immune response.[89,90] The aim of immunotherapy using antagonists of these
285 inhibitory T cell checkpoint interactions is to reactivate anti-tumour immunity. PDL1 (B7-H1)
286 is a ligand present on antigen presenting cells (APCs), including tumour cells that interacts
287 with its receptor (PD-1) on T cells and inhibits T cell effector functions. PD-1 and CD8 positive
288 effector T cell population are thought to be the tumour reactive subset responsible for anti-
289 tumour immunity.[91] There is limited knowledge of the spatial or functional heterogeneity of
290 tumour infiltrating lymphocyte (TIL) populations and the T cell checkpoint ligand-receptor
291 interactions within solid tumours.
292
293 Recent randomised trials have shown activity of PD1 and PDL1 targeting antibodies in
294 squamous and non-squamous NSCLC.[92-96] In most instances these agents have shown
295 greater activity in patients whose tumour expresses PDL1 when tested using IHC. However
296 durable responses are seen in patients without PDL1 expression. This is unsurprising given
297 the technical and spatial heterogeneity of PDL1 expression in NSCLC, which hampers its use
298 as a predictive biomarker.[97-99] Studies of the expression of PDL1 on APCs in NSCLC are also
299 contradictory with respect to any correlation with tumour infiltration of the effector CD8
300 positive T cells. [89,97,98]
301
302 Regulation of PDL1 expression is complex and controlled by both cell intrinsic and cell
303 extrinsic factors.[100] This means that oncogene driven expression of PDL1 can result in
304 increased expression in the absence of significant underlying immunogenicity.[101] This
305 underlying immunogenicity is thought to be a result of non-synonymous SNVs which
306 generate neoantigens, mutated proteins, recognised by the TIL population.[102,103] The
307 number of neoantigens harboured by a tumour could act as a potential biomarker for
308 immunotherapy although there are technical challenges inherent with such complex

assessments. Recent data also suggest that neo-antigen intratumour heterogeneity may also be associated with altered checkpoint inhibitor response, which may further complicate the use of such assays in a clinical setting.[104]

The advent of immunotherapy presents additional challenges for molecular diagnostics in NSCLC. Although IHC for PDL1 can be performed on the small samples often used in lung cancer diagnostics there is the risk of significant sampling bias because of ITH. The dynamic nature of PDL1 gene expression,[105,106] means a contemporaneous sample obtained by repeat biopsy may be the most accurate adding additional burden and expense to current clinical pathways. Characterisation of neo-antigens as a potential biomarker would require sufficient tumour DNA for WES and carries significant expense but given the cost of these therapies would be justified if the assay were sufficiently predictive. However neo-antigen prediction algorithms are still in their infancy and evidence suggests that there are a proportion of patients who derive no clinical benefit from checkpoint inhibitor therapy, yet have tumours with a neo-antigen burden above thresholds associated with sensitivity and conversely patients with low neo-antigen burden who benefit.

Molecular diagnostics in practice: The United Kingdom National Lung Matrix Trial

The Cancer Research UK Stratified Medicine Programme 2 (SMP2) screens samples from advanced NSCLC patients using NGS for known drivers that are considered clinically actionable. The aim of SMP2 is to establish high-throughput and quality genomic screening at a national level in the UK. Based on these results, patients are recruited to The National Lung Matrix Trial (NLMT) (NCT02664935) a phase II umbrella study with both targeted therapy and immunotherapy arms for patients who have progressed on first line therapy.[107] In comparison, the Lung-MAP (NCT02154490) and SAFIR02 Lung trial (NCT02117167) are umbrella studies, outside of the UK, for patients with NSCLC where recruitment is preceded by molecular stratification (Table 1).

SMP2 molecular pathology workflow utilises DNA from excess diagnostic biopsy tissue. Sections are sent from the referring clinical site and extracted by one of three central technology hubs. Samples with sufficient amounts of DNA (>50ng) are then analysed using a custom 28-gene targeted NGS panel. Having successfully screened over 1000 patients, patterns of mutation and prevalence are emerging across the genomic and clinical data.

Preliminary analysis indicates prevalence and distribution of SNVs consistent to published reports, including 31.6% KRAS (of which 19.7% show concomitant STK11 mutation) and 15.1% EGFR mutations in patients with adenocarcinoma. Over the past year, SMP2 has led to the recruitment of over 60 patients to the NLMT. A number of detailed audits have identified areas of improvement along the SMP2 pathway; from patient recruitment, to sample preparation and result analysis.

Whilst utilising excess DNA from the FFPE diagnostic biopsy has significant advantage for patients and clinical workload (as repeat biopsies are not required), only 70% of samples sent have sufficient DNA to enter the sequencing pipeline. This is in part due to FFPE blocks being exhausted during the diagnostic process and a general reduction in the size of diagnostic cores over time. Consequently the minimum number of sections has since been increased to ensure enough DNA is obtained up front. Some recruiting centres quantify DNA upfront, which allows a faster feedback loop if insufficient DNA is present. Sites can then obtain additional sample from the diagnostic block or through re-biopsy, if appropriate. However, differences in quantification methodology between local clinical centres and central technology hubs have led to samples being sent with less than the required 50ng, resulting some of these samples failing quality control metrics prior to sequencing. As a result changes in extraction methods and a standardized DNA concentration have been introduced.

Unique to NLMT is the need to determine wild type status of some genes for eligibility to certain arms. Patients recruited to the CDK4/6 inhibitor palbociclib arm must have wildtype retinoblastoma 1 protein (RB1) in addition to deficiencies in cell cycle regulation. The determination of wildtype status requires a pre-sequencing assessment of tumour cellularity to determine appropriate sequencing depth. However there can be significant discordance between pathologist assessments of this.[108] Clearer guidance and online training should ensure more concordance for visual assessment, whilst digital solutions may provide a useful alternative. A number of computational methods exist to assess tumour purity and control for both stromal cell admixture and cancer cell ploidy in DNA samples from next generation sequencing data.[109,110]

Extremes of GC nucleotide content in certain genes (RB1 and FGFR3) can result in an increased number of sequencing failures. Additional probe coverage in the targeted panel

377 and correction for GC content in the data processing stage will improve results for these
378 difficult to sequence regions. Following these incremental improvements at each step of the
379 molecular pathology workflow we have shown that the number of successfully sequenced
380 samples that would allow recruitment to the NLMT increased and there has also been an
381 increase in identification of potentially actionable mutations that would permit recruitment
382 to trials other than the NLMT.
383
384 **Future solutions**
385
386 **Understanding tumour heterogeneity and cancer evolution**
387 At present the technical limitation of the small, and potentially low tumour cellularity NSCLC
388 samples, obtained from bronchoscopic and EBUS-TBNA samples means that the main
389 challenge facing clinicians and pathologists is the need for ever greater amounts of
390 information from diminishing amounts of tissue. It is therefore imperative that the quality of
391 diagnostic samples in the advanced NSCLC setting is of the highest order. How best to
392 achieve this represents a challenge for health service providers that has received very little
393 attention thus far. However the spectre of ITH and cancer evolution means that sampling
394 bias and the presence of subclonal driver mutations, causing resistance to therapy, are likely
395 to hinder clinical benefit of targeted therapeutics.[111,112] The UK Lung TRAcking Cancer
396 Evolution through Therapy trial (NCT01888601) is currently characterizing the extent of ITH
397 in early surgically resected NSCLC and with longitudinal follow-up aims to determine the
398 origins of tumour subclones contributing to relapse.[17] There is evidence from other tumour
399 types of parallel evolution, acquisition of mutations in the same gene or signalling pathway
400 in distinct subclones, that may highlight an 'evolutionary bottle neck' that could be an
401 Achilles heel for subsequent cancer therapy.[113-115] Clonal analyses of a drug target and
402 putative resistance events, whether they are present in all tumor cells or only a proportion,
403 may affect the response rate and progression free survival times on targeted therapy and
404 this is being addressed in clinical trials including the DARWIN studies (NCT02314481,
405 NCT02183883). Ultimately it may be that only through 'warm' autopsy studies, where
406 subclonal phylogenetic structures can be determined through sampling multiple sites of
407 disease, that effective strategies to forestall cancer evolution can be elucidated.[116]
408
409 **Circulating biomarkers**
410 The use of minimally invasive methods to detect mutations in circulating cell-free DNA

411 (cfDNA) or 'liquid biopsies' offers the potential to obtain a mutation call in a patient where
412 an invasive biopsy may not be feasible. As tumour DNA from all sites of disease has the
413 potential to enter the blood stream it may also be a better reflection of tumour
414 heterogeneity than a single biopsy.[117,118] The use of cfDNA to detect resistance mechanisms
415 in patients treated with EGFR TKIs, often prior to radiographic progression, has been
416 demonstrated.[45,119,120] This has resulted in the development and approval of a commercially
417 available assay of cfDNA in plasma that can detect a spectrum of EGFR mutations in
418 including the T790M mutation amenable to targeting with third generation TKIs.
419
420 Circulating tumour cells (CTCs) are tumour cells that can be isolated from the peripheral
421 blood and are a complementary circulating biomarker to cfDNA. CTCs are a versatile tool,
422 cell enumeration can be prognostic, immunohistochemistry permits further characterization,
423 single cell DNA or RNA sequencing is possible and tumour xenografts can be generated to
424 assess drug response.[121-125] However at present the complexity of separation from other
425 cells in the peripheral circulation and the need to process samples promptly for functional or
426 genomic studies results in greater expense in comparison to cfDNA analysis. Circulating
427 biomarkers will make a significant impact on cancer management in the near future and
428 readers are directed to more extensive reviews focusing on CTCs, cfDNA and other
429 circulating nucleotides.[121,126-129]
430
431 **Conclusion**
432 The challenges for molecular diagnostics in NSCLC are largely paralleled across other tumour
433 types. Resolving these issues will require technology improvements in addition to a greater
434 understanding of tumour biology. The logistical challenges of implementing the next
435 generation of molecular diagnostics into clinical practice are equally as challenging. Clinical
436 governance, information technology infrastructure, data storage, pathways in sample
437 processing and training and professional developments in histopathology, respiratory
438 medicine and oncology will need investment. With these great challenges comes significant
439 opportunity to improve the success rate and efficiency of drug development in NSCLC and
440 ultimately patient outcomes.
441
442 Conflicts of interest: None

Figure 1: Summary of the key technical, logistical and biological challenges for molecular testing in NSCLC.

Figure 2: Pie charts showing the approximate distribution of clinically relevant driver mutations identified to date in individuals with NSCLC. The genomic variants shown are potentially clinically actionable variants.[130] The proportions presented are based on estimates from the referenced studies and data sources, including the Stratified Medicine Programme 2 (unpublished data).[2,3,85,131] These studies examine SNVs, copy number variants and gene fusion products using different sequencing technologies and sequencing depth resulting in inter-study variation and therefore the data is presented in aggregate form and represents an approximation. CCGA (Cell cycle genomic aberration); loss of Cyclin-Dependent Kinase Inhibitor 2A or amplification of Cyclin-Dependent Kinase 4 or Cyclin D1 in the presence of wildtype RB1.[107] EGFR, Epidermal growth factor receptor; LKB1, Liver Kinase B1; ALK, anaplastic lymphoma kinase; MET, MET Proto-Oncogene, Receptor Tyrosine Kinase; FGFR, fibroblastic growth factor receptor; NRAS, neuroblastoma RAS viral (v-ras) oncogene homolog; DDR2 Discoidin Domain Receptor Tyrosine Kinase 2; AKT1, v-akt murine thymoma viral oncogene homolog 1; PTEN, Phosphatase And Tensin Homolog; PIK3CA, phosphoinositide-3-kinase, catalytic, α polypeptide; BRAF, v-raf murine sarcoma viral oncogene homolog B1; ERBB2, human epidermal growth factor receptor 2; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; TP53, Tumor Protein P53.

Table 1: Comparison of molecularly stratified umbrella studies in NSCLC. PFS = progression free survival, ORR = objective response rate, R = randomised, NR = non-randomised, SCC = squamous cell carcinoma, CCGA = cell cycle genomic aberration.

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Technical

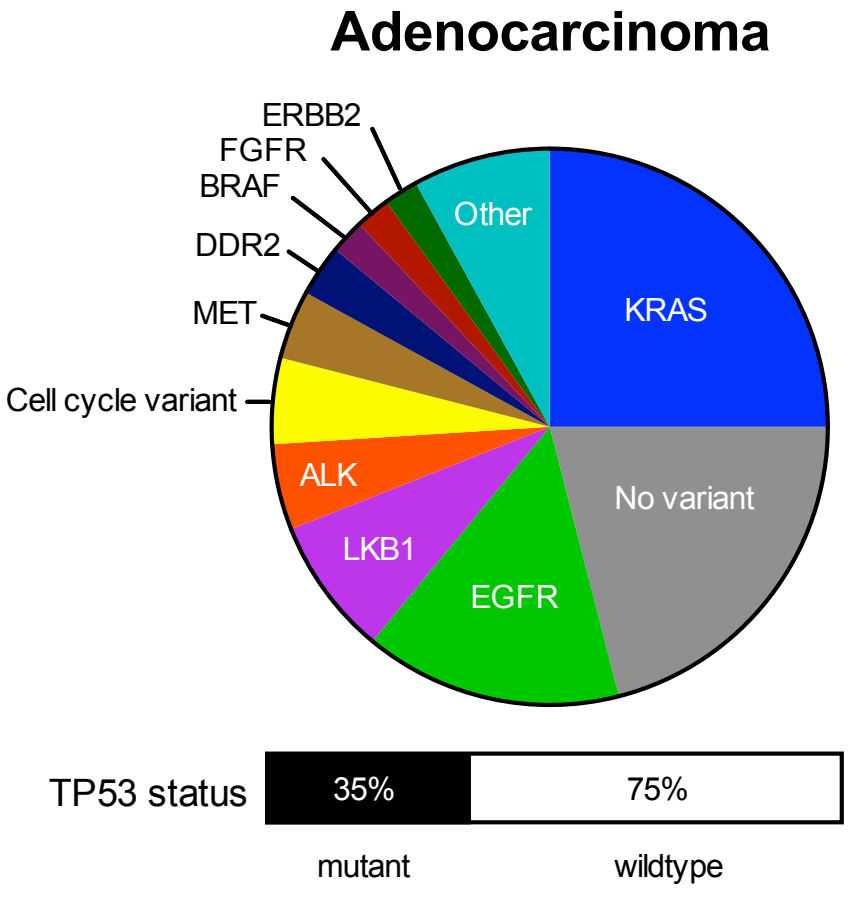
- Novel biopsy techniques (eg EBUS) generating smaller samples with diminished tumour cellularity
- Multiple tests with the potential for discordant results (eg IHC vs FISH for ALK mutation)
- Technology-specific failures due to differences in sensitivity/known artefacts (eg sequencing through repeats/high GC areas)
- Quality assurance of genomic medicine despite across multiple platforms and data analysis algorithms

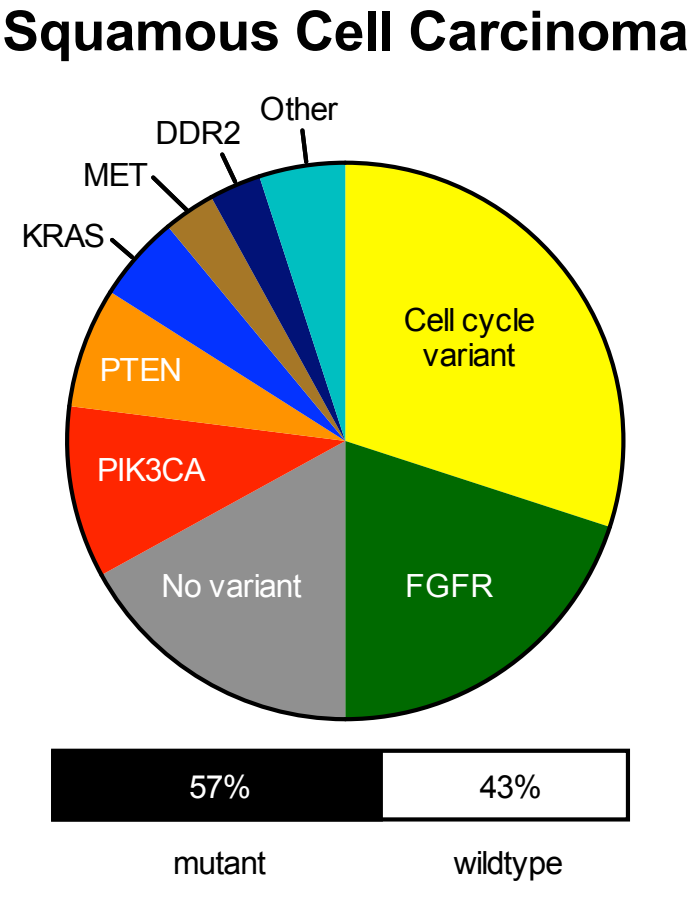
Logistical

- Turnaround time of assays in a clinically relevant timeframe
- Desirability of centralised vs distributed/local testing approaches
- Education and training of laboratory and clinical staff n new technologies
- Distillation of high volume data into useful standardised reports usable by clinicicans
- Computational and data storage capacity for NGS within a healthcare system

Tumour Biology

- Diversity of molecular subgroups within NSCLC
  - inter-patient heterogeneity
- Intra-tumour heterogeneity
  - sampling bias
  - differential responses
- Cancer evolution and resistance in response to treatments
  - need for longitudinal sampling
- Evolving treatment paradigms
  - immuno-oncolog & new biomarkers (eg PDL-1, neoantigen load)
- Increasing complexity of detectable genomic changes in cancer
  - eg epigenetic changes & non-coding variants


Adenocarcinoma
ERBB2
FGFR
BRAF
DDR2
MET
Cell cycle variant
ALK
LKB1
EGFR
No variant
KRAS
Other
TP53 status
35%
75%
mutant
wildtype


Squamous Cell Carcinoma
Other
DDR2
MET
KRAS
PTEN
PIK3CA
No variant
FGFR
Cell cycle variant
57%
43%
mutant
wildtype

Study	Line	Phase	POM	Molecular subgroups	Location
National Lung Matrix Trial	2nd or later	NR Phase II	PFS ORR	AKT, PIK3CA/PTEN, TSC, LKB1, KRAS, NRAS, NF1, MET, ROS1, EGFR (T790M), CCGA, immunotherapy	UK
SAFIR_02 Lung Study	1st line maintenance	R Phase II	PFS	mTOR, AKT, FGFR, HER2, EGFR, MEK, immunotherapy	France
Lung MAP	2nd or later (SCC)	NR Phase II	PFS ORR	PIK3CA, FGFR, CCGA, immunotherapy	USA
Darwin I/II	1st or later	NR Phase II	PFS	EGFR, HER2, ALK, RET, BRAF, immunotherapy	UK