

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

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23 Summary:

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

35

## 36 **Historical overview of lung cancer diagnostics**

37

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

50

## 51 **Identification of tumour histology**

52

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

62

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

70 synonymous with adenocarcinoma, is expressed in only 80-90% of cases and is commonly  
71 expressed in neuroendocrine tumours.<sup>(14,16)</sup> Immunohistochemistry can only be meaningfully  
72 interpreted in a detailed morphological context.

73

#### 74 **Sampling challenges in advanced NSCLC**

75

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

82

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

94

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

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

111

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

123

124

### 125 **Current challenges in molecular diagnostics for EGFR mutation analysis in clinical practice**

126

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

135

### 136 **Development of diverse mechanisms of resistance and selection of resistant clones in** 137 **response to treatment**

138 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.<sup>(36)</sup> The T790M mutation in exon 20  
141 results in resistance to these therapies.<sup>(37)</sup> 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.<sup>(38-40)</sup> 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.<sup>(41,42)</sup> 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.<sup>(40)</sup> 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.<sup>(43)</sup> 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.<sup>(44,45)</sup>

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.<sup>(46)</sup> 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.<sup>(47,48)</sup> 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 technologi<sup>(49)</sup>s 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.<sup>(50)</sup> 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.<sup>(51)</sup> 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.<sup>(52-54)</sup>

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.<sup>(55)</sup> Loss  
184 of the EGFR mutation was not a mechanism seen in seminal studies.<sup>(43)</sup> 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.<sup>(56,57)</sup> 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.<sup>(58)</sup> 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.<sup>(59-61)</sup> 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.<sup>(59,62,63)</sup> Subsequently other rare fusion genes have been identified  
203 involving ROS1, with similar exquisite sensitivity to kinase inhibition,<sup>(64)</sup> but also RET and  
204 NTRK where objective response rates are lower.<sup>(65,66)</sup>

205

### 206 **ALK fusion gene detection**

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

217

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

227

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

241 ALK (and other) rearrangements in either a targeted panel or a WES or WGS approach.<sup>(73,74)</sup>  
242 <sup>(72)</sup>

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.<sup>(75)</sup> 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.<sup>(76-78)</sup> 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.<sup>(79-82)</sup> 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.<sup>(83,84)</sup> 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.<sup>(85)</sup> 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.<sup>(86)</sup> 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,<sup>(87)</sup> and some advocate that modifications to existing paradigms in drug



275 development are required in the era of genomic studies and precision medicine.<sup>(88)</sup> 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.<sup>(89,90)</sup> 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.<sup>(91)</sup> 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.<sup>(92-96)</sup> 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.<sup>(97-99)</sup> 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.<sup>(89,97,98)</sup>

301

302 Regulation of PDL1 expression is complex and controlled by both cell intrinsic and cell  
303 extrinsic factors.<sup>(100)</sup> This means that oncogene driven expression of PDL1 can result in  
304 increased expression in the absence of significant underlying immunogenicity.<sup>(101)</sup> 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.<sup>(102,103)</sup> 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

309 assessments. Recent data also suggest that neo-antigen intratumour heterogeneity may also  
310 be associated with altered checkpoint inhibitor response, which may further complicate the  
311 use of such assays in a clinical setting.<sup>(104)</sup>

312

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

325

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

327

328 The Cancer Research UK Stratified Medicine Programme 2 (SMP2) screens samples from  
329 advanced NSCLC patients using NGS for known drivers that are considered clinically  
330 actionable. The aim of SMP2 is to establish high-throughput and quality genomic screening  
331 at a national level in the UK. Based on these results, patients are recruited to The National  
332 Lung Matrix Trial (NLMT) (NCT02664935) a phase II umbrella study with both targeted  
333 therapy and immunotherapy arms for patients who have progressed on first line therapy.<sup>(107)</sup>  
334 In comparison, the Lung-MAP (NCT02154490) and SAFIRO2 Lung trial (NCT02117167) are  
335 umbrella studies, outside of the UK, for patients with NSCLC where recruitment is preceded  
336 by molecular stratification (Table 1).

337

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

343 Preliminary analysis indicates prevalence and distribution of SNVs consistent to published  
344 reports, including 31.6% KRAS (of which 19.7% show concomitant STK11 mutation) and  
345 15.1% EGFR mutations in patients with adenocarcinoma. Over the past year, SMP2 has led  
346 to the recruitment of over 60 patients to the NLMT. A number of detailed audits have  
347 identified areas of improvement along the SMP2 pathway; from patient recruitment, to  
348 sample preparation and result analysis.

349

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

363

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

374

375 Extremes of GC nucleotide content in certain genes (RB1 and FGFR3) can result in an  
376 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.<sup>(111,112)</sup> 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.<sup>(17)</sup> 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.<sup>(113-115)</sup> 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.<sup>(116)</sup>

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.<sup>(117,118)</sup> The use of cfDNA to detect resistance mechanisms  
415 in patients treated with EGFR TKIs, often prior to radiographic progression, has been  
416 demonstrated.<sup>(45,119,120)</sup> 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.<sup>(121-125)</sup> 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.<sup>(121,126-129)</sup>

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.<sup>(130)</sup> The proportions presented are based on estimates from the referenced studies and data sources, including the Stratified Medicine Programme 2 (unpublished data).<sup>(2,3,85,131)</sup> 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.<sup>(107)</sup> 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,  $\alpha$  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

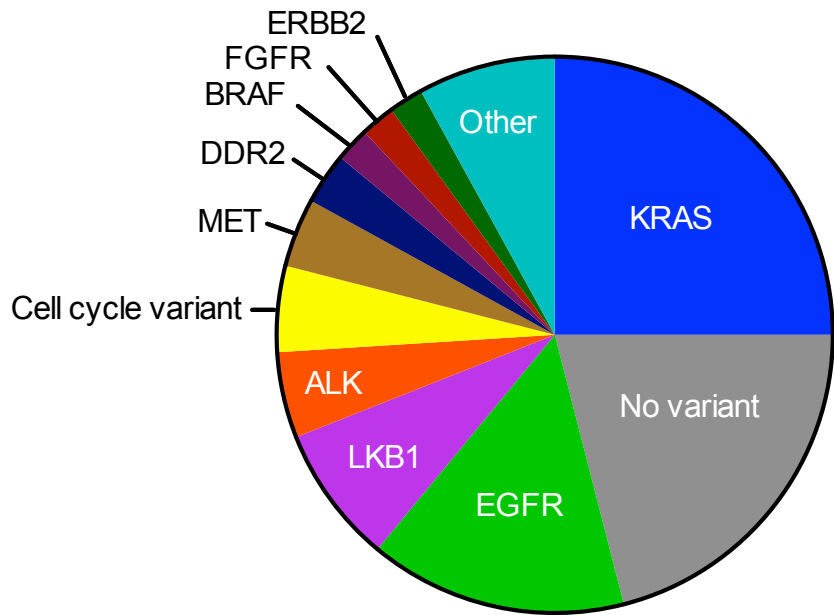
- Turnaround time of assays in a clinically relevant timeframe
- Desirability of centralised vs distributed/local testing approaches
- Education and training of laboratory and clinical staff in new technologies
- Distillation of high volume data into useful standardised reports usable by clinicians
- 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-oncology & new biomarkers (eg PDL-1, neoantigen load)
- Increasing complexity of detectable genomic changes in cancer
  - eg epigenetic changes & non-coding variants



# Adenocarcinoma



TP53 status

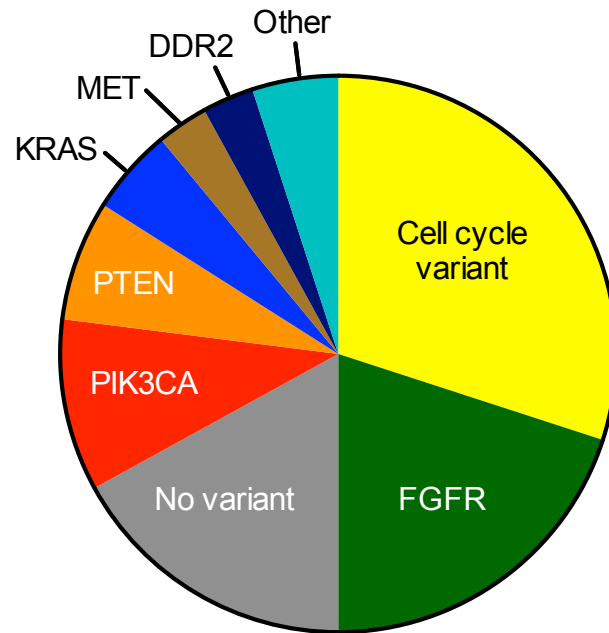
35%

mutant

75%

wildtype

# Squamous Cell Carcinoma



57%

mutant

43%

wildtype

<b>Study</b>	<b>Line</b>	<b>Phase</b>	<b>POM</b>	<b>Molecular subgroups</b>	<b>Location</b>
National Lung Matrix Trial	2 <sup>nd</sup> 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	2 <sup>nd</sup> or later (SCC)	NR Phase II	PFS ORR	PIK3CA, FGFR, CCGA, immunotherapy	USA
Darwin I/II	1 <sup>st</sup> or later	NR Phase II	PFS	EGFR, HER2, ALK, RET, BRAF, immunotherapy	UK

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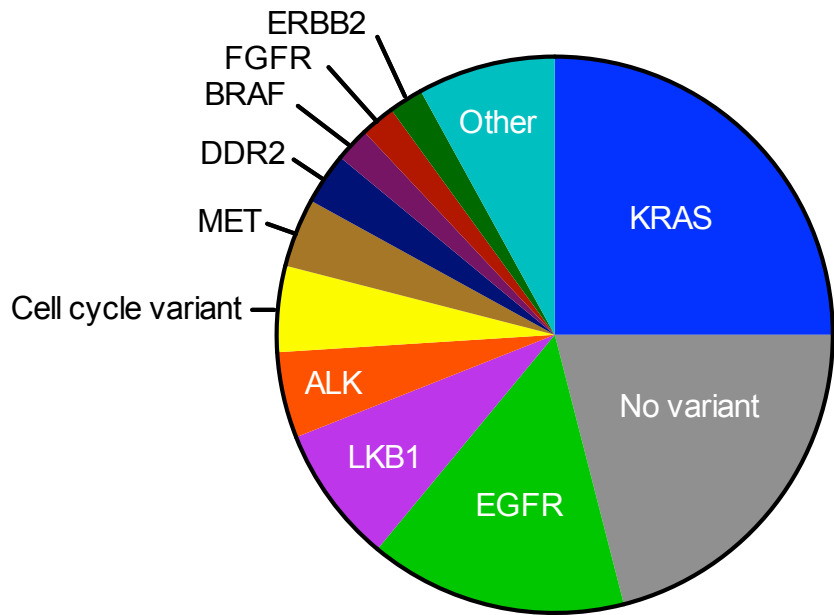
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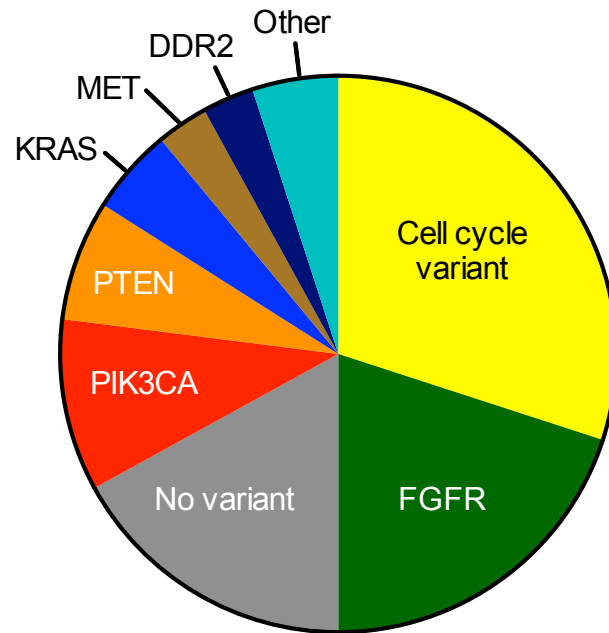
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