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Circulating Tumor DNA as a Liquid Biopsy: Current Clinical Applications and Future Directions: Page 2 of 3

By Kimberly M. Komatsubara, MD and Adrian G. Sacher, MD, MMSc

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Abstract / Synopsis:

Tumor genomic sequencing has become part of routine oncology practice in many tumor types, in order to identify potentially targetable mutations and to personalize cancer care. Plasma genotyping via circulating tumor DNA analysis is a noninvasive and rapid alternative method of detecting and monitoring genomic alterations throughout the course of disease. Multiple assays have been developed to date, each with different test characteristics and degrees of clinical validation. Here we review the clinical data supporting these different plasma genotyping methodologies, and present a practical approach to the interpretation of the results of these tests. While the clinical application of plasma genotyping has been most extensively validated in the metastatic setting—for the detection of targetable alterations at the time of initial diagnosis or disease progression—this technology holds significant promise across many tumor types and stages of disease. We will also review emerging applications of plasma genotyping that are currently under clinical investigation.

Guide to Interpretation of Plasma Genotyping Results

With a multitude of commercial assays now available, plasma genotyping is quickly emerging as a routine tool in the armamentarium available for the diagnosis and management of cancer. The clinical utility of plasma genotyping for directing patient care has been most extensively studied at the times of initial diagnosis and disease progression in advanced lung cancer, where molecular testing results directly impact treatment decision making. While tumor tissue genotyping is considered the standard choice for detection of potentially targetable alterations, plasma genotyping may have a role, particularly in settings where either a tissue biopsy is technically infeasible or tissue is inadequate to perform routine genotyping.

Oncology (Williston Park). 31(8):618–627.

Table 1. Comparison of Selected ctDNA Assays for Detection of *EGFR* Mutations

Platform	Key Test Characteristics	Validation Study (Prospective vs Retrospective)	Sensitivity (for *EGFR* Mutations)	Specificity (for *EGFR* Mutations)	Reference(s)
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	Detects known SNV mutations	Retrospective	61.4% (*EGFR* T790M)	78.6% (*EGFR* T790M)	[15,16]
	Semi-quantitative				
Scorpion-ARMS	Detects known SNV mutations	Retrospective	61.8%–85.7% (*EGFR* exon 19 del and L858R)	94.3%–100% (*EGFR* exon 19 del and L858R)	[2,17]
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ARMS = amplification refractory mutation system; BEAMing = beads, emulsion, amplification, and magnetics; ctDNA = circulating tumor DNA; ddPCR = digital droplet polymerase chain reaction; del = deletion; FDA = US Food and Drug Administration; NGS = next-generation sequencing; SNV = single nucleotide variant.

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Table 2. Interpretation of ctDNA Testing Results

ctDNA Test Result	Is Tumor Shedding ctDNA?	Interpretation	Clinical Implication
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No mutations detected	?	Nonshedding tumor? Or true-negative test?	Reflex tumor biopsy

ctDNA = circulating tumor DNA; del = deletion; EGFR = epidermal growth factor receptor; TKI = tyrosine kinase inhibitor.

Table 2. Interpretation of ctDNA Testing Results

Plasma genotyping at initial diagnosis

Careful consideration of the biology underlying ctDNA shed and an understanding of the characteristics of the particular assay being used are required to accurately interpret ctDNA test results. As discussed in the aforementioned studies, validated ctDNA assays are characterized by exquisitely high specificity and positive predictive values. As a result, a driver mutation detected by plasma genotyping can be interpreted as a true-positive result. The ability to detect a driver mutation also implies that a tumor is shedding ctDNA at a detectable level. Thus, any negative result in this setting can also be interpreted as a true negative (Table 2). For example, the absence of an *EGFR* mutation on plasma genotyping, but detection of a *KRAS* ctDNA mutation, can be interpreted as a true-negative result and a true-positive result, respectively.

Interpretation is more complex in the setting where no genomic alterations are detected. In such a case, either the tumor does not possess any of the mutations of interest, or the tumor is not shedding any ctDNA—meaning that the result might be a false

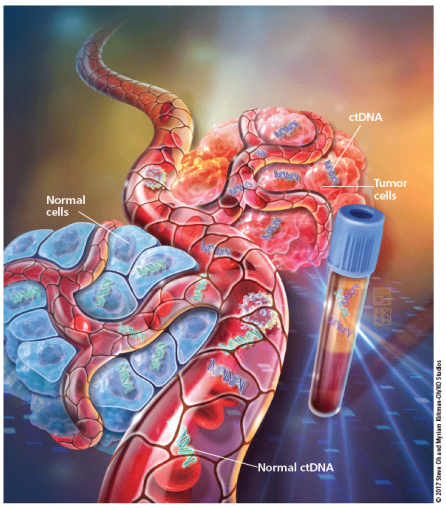

Figure. Circulating Tumor DNA as a Liquid Biopsy—Both normal cells and tumor cells release cell-free DNA (cfDNA) into the peripheral blood. Tumor-derived cfDNA, or circulating tumor DNA (ctDNA), can be detected and utilized as a "liquid biopsy" to noninvasively assess for tumor molecular alterations.

Figure. Circulating Tumor DNA as a Liquid Biopsy

negative. Furthermore, while the majority of plasma genotyping assays have near-perfect specificity, sensitivity generally ranges from 70% to 80%, resulting in false-negative tests due to inherent analytic test factors. Thus, a tumor biopsy for confirmatory genotyping should be considered when plasma genotyping results are entirely negative (see Table 2).

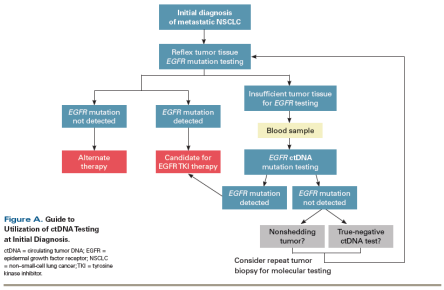

Figure A

Plasma genotyping for resistance mutations

The ability to detect resistance mutations at disease progression is another property of noninvasive testing with plasma genotyping that may be particularly valuable clinically. The detection of *EGFR* T790M mutations by ctDNA analysis is a key example of this clinical application. Multiple studies in this particular setting have reported lower specificities for the detection of *EGFR* T790M when plasma genotyping has been compared with tissue genotyping. Thress et al reported sensitivities of 73% and 81% and specificities of 67% and 58% when using the cobas and BEAMing platforms, respectively, for the detection of *EGFR* T790M. In their analysis they concluded that many of the “false-positive” results were more likely explained by tumor heterogeneity of resistance mutations, rather than being due to a lack of specificity of the assay.[27] Interestingly, subsequent studies have demonstrated that patients treated with osimertinib based on a positive plasma test for the *EGFR* T790M mutation have outcomes similar to those of patients who are treated based on tissue genotyping results.[20] The recent confirmatory AURA3 study, which randomized patients with T790M-positive NSCLC who had progressed following treatment with at least one EGFR kinase inhibitor to either osimertinib or platinum-based chemotherapy, demonstrated similar outcomes for patients who were T790M-positive by ctDNA testing and those positive by tumor testing, supporting the feasibility of using plasma genotyping in this clinical setting.[28] Thus, with regard to resistance mutation detection, plasma genotyping may be useful to confirm the presence of a targetable resistance mutation such as *EGFR* T790M. Because the sensitivity of plasma genotyping assays is not perfect, however, a negative ctDNA test should still be followed by a reflex tumor biopsy when feasible, since the latter may identify alternate mechanisms of resistance, such as transformation of disease or complex alterations, which are not as easily identified by plasma genotyping.

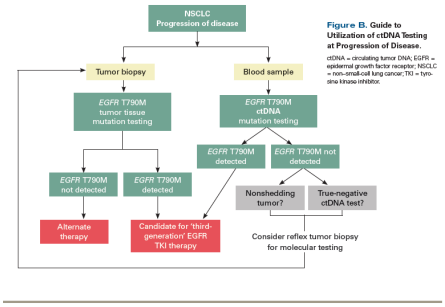

Figure B

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Introduction

Modern targeted therapies have greatly advanced the treatment of many solid tumors. The rational use of these agents requires optimal strategies for the rapid and accurate detection of targetable genomic alterations, both upon initial diagnosis and when acquired resistance to targeted therapies develops. While tissue genotyping has traditionally been considered the standard of care for identifying such genomic alterations, this methodology is limited by often inaccessible or insufficient tumor tissue, and by the risks associated with undergoing serial tumor biopsies. In non–small-cell lung cancer (NSCLC), for example, where molecular testing to guide initial treatment decision making is considered standard of care, clinical studies show that up to 10% to 20% of biopsies are inadequate for molecular testing due to insufficient tissue or amplifiable DNA.[1,2] Furthermore, tumor biopsies are limited by tumor heterogeneity, particularly in the setting of disease resistance, and thus may yield false-negative results.

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Table 2. Interpretation of ctDNA Testing Results

Plasma genotyping is an emerging technology that can noninvasively and rapidly detect and monitor genomic alterations in cancer patients over time, and which has the potential to overcome some of the limitations associated with tissue genotyping. To date, multiple platforms for plasma genotyping have been tested and validated to various degrees; each has different advantages and limitations, complicating the interpretation and routine integration of these tests into clinical practice.

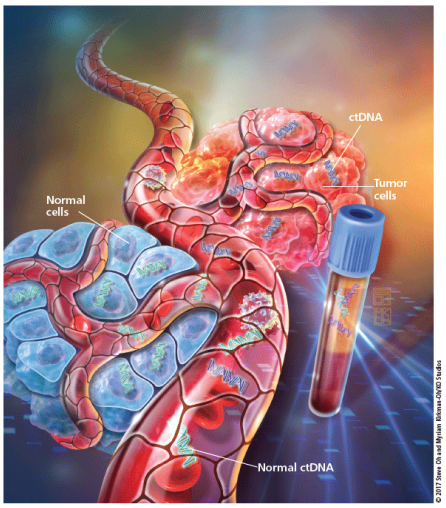

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Figure. Circulating Tumor DNA as a Liquid Biopsy

Biology of Cell-Free DNA

The presence of cell-free DNA (cfDNA) in the plasma was first described in 1948 by Mandel and Metais.[3] It was not until years later that tumor-derived cfDNA—also called circulating tumor DNA (ctDNA)—was discovered, stemming from the finding that cancer patients had higher levels of plasma cfDNA than normal controls.

[4,5] The exact mechanism by which DNA is released into the peripheral blood has not been well described; however, it is thought to occur through multiple mechanisms, including tumor cell apoptosis, necrosis, and extracellular vesicle secretion.[6-8] In contrast to genomic DNA, ctDNA is highly fragmented, with most DNA fragments measuring approximately 150 bp in length; this supports the hypothesis that cfDNA originates through cell necrosis or apoptosis.[9,10] Also, peripheral cfDNA exists in low concentrations, with the majority of studies reporting absolute cfDNA levels of less than 100 ng/mL[11]—and only a fraction of this total cfDNA (< 1% of total cfDNA) is actually tumor-derived.[9,12] Thus, highly sensitive methodologies are required to detect ctDNA (Figure).

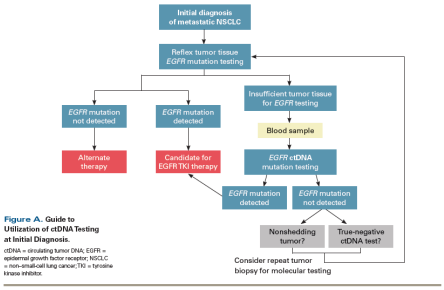

Figure A

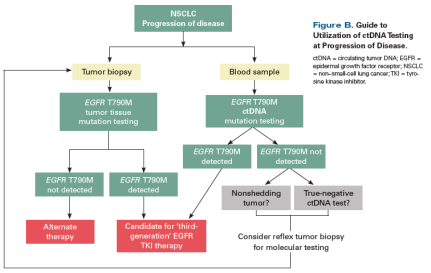

Figure B

The concept of ctDNA "shed" is critical to the understanding and interpretation of plasma genotyping assay results, since even the most sensitive assays may not detect a mutation if a tumor is not shedding ctDNA. The degree to which a tumor releases DNA into the peripheral circulation determines the concentration of ctDNA that may be detected in the blood. Factors associated with increased ctDNA shed include mitotic rate, cell death, and tumor vascularization. The extent of metastatic disease burden and sites of metastasis have also been associated with detection of increased ctDNA.[6,7] In particular, the presence of metastasis to the liver or bone has been associated with higher levels of ctDNA in clinical studies.[13]

Current Assays and Validation

A variety of potential testing platforms for plasma genotyping have been utilized in the past. Each platform has been validated to a different degree and exhibits unique capabilities and test characteristics. Traditional DNA detection methods, such as Sanger sequencing, have generally lacked the sensitivity needed to detect the low levels of ctDNA present in the peripheral blood. Thus, more recent assays have utilized an allele-specific quantitative polymerase chain reaction (PCR)-based platform to enrich for mutant DNA, or have used massive parallel sequencing with a next-generation sequencing (NGS) platform. Most currently validated ctDNA assays have been compared against tissue genotyping. The specificities reported for these tests are consistently high, ranging between 90% and 100%, but their sensitivities are more variable, ranging between

30% and 85%, depending on the testing methodology (Table 1). While few prospective validation studies have been carried out to date, retrospective analysis of samples collected from patients enrolled in prospective clinical trials is a reliable alternative.

Approved assays

The Roche cobas EGFR Mutation Test v2 is the only ctDNA assay that is currently approved by the US Food and Drug Administration (FDA) for the detection of *EGFR*-sensitizing and resistance mutations in NSCLC. This platform was originally approved by the FDA for the detection of *EGFR*-sensitizing mutations in tissue. Recently, this approval has been extended to include plasma genotyping for both *EGFR*-sensitizing mutations and the T790M resistance mutation. The approval of the plasma genotyping assay was based on data from the retrospective analysis of paired plasma and tissue samples from 517 NSCLC patients screened for the ENSURE study, which compared treatment with cisplatin/gemcitabine vs erlotinib in *EGFR*-mutant NSCLC patients. The cobas assay demonstrated moderate sensitivity and high specificity—76.7% (range, 70.5%–81.9%) and 98.2% (range, 95.4%–99.3%), respectively—for the detection of *EGFR*-sensitizing mutations.[14] The approval for inclusion of *EGFR* T790M was based on a retrospective analysis of plasma samples from *EGFR*-mutant NSCLC patients with acquired resistance to initial kinase inhibitor therapy who were enrolled in the early-phase trials (AURA and AURA2) of the third-generation epidermal growth factor receptor (EGFR) kinase inhibitor osimertinib. When compared with an NGS plasma genotyping assay, the sensitivity and specificity of the cobas assay for detection of *EGFR* T790M were 91.5% and 91.1%, respectively; however, when compared with tissue genotyping, sensitivity and specificity were lower, at 61.4% and 78.6%, respectively.[15,16] The discordance of these results was hypothesized to be related to the heterogeneity of the presence of resistance mutations in tumor tissue.

PCR-based assays

Many of the earliest attempts at ctDNA genotyping utilized the Scorpion amplification refractory mutation system (Scorpion-ARMS) platform, which uses allele-specific primers with fluorescent probes to amplify target DNA alleles. Kimura et al evaluated the test characteristics of ctDNA analysis using a Scorpion-ARMS platform, comparing it against tissue genotyping in NSCLC patients treated with gefitinib. They demonstrated that cfDNA analysis had the ability to detect

EGFR mutations with a sensitivity of 85.7% and a specificity of 94.3%.[17] Similar results have been reported in several other retrospective validation studies that used Scorpion-ARMS technology to assess plasma *EGFR* mutations in the NSCLC population.[2,18]

Newer PCR methodologies, such as beads, emulsion, amplification, and magnetics (BEAMing) and digital droplet PCR (ddPCR), are characterized by higher sensitivity and specificity, and have the additional capability of quantitating mutant ctDNA levels. A recent prospective clinical study compared a ddPCR-based plasma assay against tissue genotyping for the detection of *EGFR* and *KRAS* mutations in patients with advanced NSCLC. This assay demonstrated 100% specificity for the detection of *EGFR*-sensitizing and *KRAS* mutations.[13] The median turnaround time for ctDNA testing results was only 3 days (range, 1–7 days), highlighting another advantage of ddPCR technology.[13] BEAMing is a highly specific and sensitive PCR methodology with quantitative capabilities. Bettegowda et al reported ctDNA test characteristics using a BEAMing platform in a cohort of 640 patients across all tumor types and stages, demonstrating variability based on stage and tumor type. Within the cohort of 206 patients with metastatic colorectal cancer, the sensitivity for detection of *KRAS* mutations was 87.2% and the specificity was 99.2%.[19] ctDNA analysis using BEAMing technology has also been retrospectively validated in studies in NSCLC to detect both *EGFR*-sensitizing and *EGFR* resistance mutations.[20,21] One limitation of both ddPCR and BEAMing is their limited ability to assess for more complex alterations, such as translocations or copy number alterations.

NGS assays

NGS technologies are capable of sequencing millions of small DNA fragments in parallel, resulting in highly sensitive and specific detection capabilities. NGS platforms also have the ability to quantify allele frequency and can detect complex alterations such as translocations and copy number changes. Using a multiplex NGS PCR platform to investigate for a panel of 50 cancer-related genes, Couraud et al reported an assay specificity of 86% to 100% and a sensitivity of 58% for the detection of all 50 genes.[22] The ability to detect complex alterations present at low allele frequencies using NGS technology was demonstrated in a study by Paweletz et al. Complex alterations—including rearrangements in *ALK, ROS1*, and *RET*; *HER2* insertions; and *MET* amplifications—were detected with 100% specificity and 77% sensitivity.[23] Several commercially available ctDNA assays, including Guardant360 (Guardant Health) and FoundationACT (Foundation Medicine), utilize NGS platforms to investigate for a panel of potentially actionable mutations. Lanman et al validated the Guardant360 platform in a study of

165 patients with advanced solid tumors and reported a sensitivity of 85% and a specificity of > 99%.[24] A subsequent large retrospective analysis of ctDNA testing results from more than 15,000 patients in whom the Guardant360 platform was used reported an accuracy of 87% when plasma genotyping was compared with historical tissue genotyping data from the Cancer Genome Atlas.[25,26]

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JOURNAL OF TUMOR

Clinical and Investigational Applications of Liquid Biopsy in Non-Small Cell Lung Cancer

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Conflict-of-interest statement: The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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ABSTRACT

Quite frequently it is difficult to obtain tissue biopsies in non-small cell lung cancer (NSCLC) patients; in some cases they are not even accessible. However, international guidelines recommend repeated biopsies prior to making a therapeutic decision. The “liquid biopsy” tries to integrate different approaches to address the use of peripheral blood as the source of molecular information through different types of materials and techniques, resolving these limitations and being a subrogate source of tumor information, offering the opportunity to increase the knowledge of the pathophysiology of lung cancer as a dynamic disease and into the process of metastatic dissemination. Circulating nucleic acids as ctDNA or exosomal RNA as well as CTCs or tumor-educated platelets offer the potential for early diagnosis, metastatic progression, stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms, and understanding metastasis development in cancer patients.

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Key words: Liquid biopsy; Non-small cell lung cancer; Circulating tumor cells; ctDNA; Exosomes

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INTRODUCTION

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In Oncology, the evaluation of response to treatment as well as accurate predictions of survival are key factors for the effective control of the disease as well as to design and develop future treatments. However, up to now, the methods we use to represent this response are relatively poor and, in the majority of cases, we can only wait for a period of time from the administration of the treatment to correlation in an image which is not always accurate in determining the extent of disease.

Additionally, quite frequently it is difficult to obtain tissue biopsies in non-small cell lung cancer (NSCLC) patients; in some cases they are not even accessible. However, international guidelines recommend repeated biopsies prior to making a therapeutic decision and it is imperative that therapeutic biomarkers are detected as early as possible so as to allow a potentially successful change in the course of treatment. The “liquid biopsy” tries to integrate different approaches to address the use of peripheral blood as the source of molecular information through different types of materials and techniques, resolving these limitations and being a subrogate source of tumor information, offering the opportunity to increase the knowledge of the pathophysiology of lung cancer as a dynamic disease and into the process of metastatic dissemination.

The study of molecular alterations in the liquid biopsy not only allows us to identify, noninvasively, candidate patients to receive the correct and more precise therapies, but it allows for closer monitoring of treatment, as plasma samples can much more easily obtained in each patient´s visit than for example an imaging test such as PET or CT[1-4]. On the other hand, several studies have shown that the ability of radiological studies and its criteria to predict the survival of cancer patients in the long term is limited[5,6].

However, the achievement of new biopsies at the time of relapse is limited due the accessibility of the metastasis, the need of invasive procedures or the quality of the biopsied specimen. Moreover, in some cases, the patient is unwilling to undergo an invasive procedure. In this sense, liquid biopsy might overcome such limitations as it is non-invasive blood test that detects circulating tumor cells (CTCs) or fragments of circulating tumor DNA (ctDNA) that are released into the blood stream from the primary tumor and from metastatic sites, being an attractive alternative for mutation search and testing.

Circulating nucleic acids as ctDNA or exosomal RNA as well as CTCs or tumor-educated platelets offer the potential for early diagnosis, metastatic progression, stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms, and understanding metastasis development in cancer patients[7-9].

Despite these components have been mainly obtained from blood, they have also been isolated from almost all body fluids (blood, serum, plasma, saliva, urine, etc)[10].

CIRCULATING TUMOR CELLS (CTCS)

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ABSTRACT

Tumor cells are shed from the primary and the metastatic sites into the bloodstream. They have been demonstrated in the blood of patients with various solid tumors and they

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have been associated with poor outcome in metastatic NSCLC patients among other tumors[10,11]. CTCs have long been considered to reflect tumor aggressiveness. In this regard, the decrease in the number of CTCs during different treatments (surgery, chemotherapy, radiotherapy, etc) has been associated with radiographic tumor response while increase in the number of cells has been correlated with tumor progression[12].

CTCs detection and characterization represent a major challenge as they may comprise both the phenotypic and genetic information of the primary tumors. As CTCs are infrequent, appearing at an estimated level of one against the background of millions (106–107) of surrounding normal peripheral mononuclear blood cells in patients with metastatic carcinomas, their detection and characterization require highly sensitive and specific methods. These methods should combine isolation and detection strategies[13]. So far, various techniques have been applied for CTC isolation and enumeration.

There is only one platform, named CellSearchTM, approved by the U.S. FDA (Food and Drug Administration) in this regard. This technology utilizes EpCAM-coated magnetic beads to isolate CTCs in several types of carcinomas in spite of limited detection efficiency. Krebs et al publicated a detection rate of 32% among patients with metastatic NSCLC disease, having ≥ 2 CTCs before chemotherapy treatment[14].

Microfluidic platforms, as the 'CTC-chip', have been shown to be capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with EpCAM-coated micropores under precisely controlled laminar flow conditions, and without requisite pre-labelling or processing of sample. These chips had a sensitivity of almost 100% patient samples analyzed, and an average purity of 52%[15].

Most importantly, the sensitivity of these EpCAM-dependent devices may by limited, due to the epithelial-mesenchymal plasticity, as CTCs lose their epithelial markers. Therefore, several EpCAM-independent isolation techniques have been tested based on their physical properties, with size-based properties (e.g., ISET, isolation by size of epithelial tumor cells) or density centrifugation steps (e.g., FICOLL)[16,17]. In this sense, a recent paper reported that CTCs could be detected in 80% of peripheral blood samples collected from 40 chemonäive, stages IIIA to IV NSCLC patients using ISET compared with 23% using CellSearchTM. A subpopulation of CTCs isolated by ISET did not express epithelial markers and circulating tumor microemboli (clusters of ≥ 3 CTCs) were observed in 43% patients using ISET but were undetectable by CellSearchTM. Authors concluded that both techniques should be performed together to allow more complete exploration of CTCs[18].

Postoperatively, the eradication or decrease of CTCs following treatment is associated with improved clinical outcomes [19-20]. A recent work has analyzed also the kinetics of CTC in a prospective study where blood samples of 56 patients for CTC analysis were obtained before and one month after surgery. Their results showed that presence of CTCs after surgery was significantly associated with early recurrence ($p = 0.018$) and a shorter disease-free survival (DFS) ($p = 0.008$)[21].

The influence of radiotherapy (RT) on the kinetic of CTC has also been reported. Dorsey et al[22] investigated the change of CTC number in patients with localized NSCLC undergoing radiation treatment. Using a telomerase-based detection assay, 65% of the 30 patients were positive for CTCs prior to treatment. CTC numbers significantly reduced after radiation (9.1 vs 0.6 CTCs/ml; $p < 0.001$). This study suggested that analyzing CTC appear to reflect response to RT in patients with localized NSCLC.

For metastatic stages, a number of studies have reviewed its prognostic significance, concluding that baseline CTC count is an independent negative prognostic factor for NSCLC. Krebs et al[14] showed median overall survival of 8.1 months and 4.3 months ($p < 0.001$) for patients with stage III or IV NSCLC using CTC cut-offs of <5 vs ≥ 5 respectively and with corresponding progression free survival (PFS) of 6.8 and 2.4 months ($p < 0.001$), using the CellSearch system. In multivariate analysis, CTC number was the strongest predictor of overall survival (OS) (HR 7.92; 95% CI 2.85–22; $p < 0.001$) exceeding the traditional risk factors of stage and performance status. Another study of 46 patients, with newly diagnosed or recurrent NSCLC, where CTC were measured at baseline in all patients, and in 23 patients CTCs were also measured before every chemotherapy cycle. A baseline CTC count of

more than eight prior to chemotherapy was a strong predictor of reduced PFS (p = 0.018) and OS (p = 0.026). However, no correlation was observed between CTCs count and tumor size after two chemotherapy cycles[23].

As a potentially useful tool for clinical practice, several studies have provided evidence between decreases in CTCs counts and radiographic response by either FDG-PET/CT or RECIST to predict DFS and OS. In a phase II clinical trial of erlotinib and pertuzumab, higher baseline CTCs counts were statistically associated with response to treatment by RECIST, and decreased CTC counts upon treatment were significantly associated with FDG-PET and RECIST response and longer PFS[24]. Nevertheless, a study subsequently published failed to demonstrate prior results, probably because of the analysis of cohort of patients with different stages and histologies, both of which can confound the interpretation of FDG uptake and CTC analysis[25].

These papers concluded that CTCs might stand as prognostic markers in cancer patients and, in the future, may help in therapy decision making, especially to select patients at a higher risk of relapse who might benefit from adjuvant therapies.

The development of personalized medicine with several targeted therapies has been associated with an increased interest in CTC isolation and characterization. EGFR mutations analysis has been examined in CTC from advanced NSCLC patients, identifying both sensitizing mutations or the T790M resistant mutation. Marchetti et al[26] identified for the first time, using the CellSearch System coupled with next-generation sequencing (NGS), EGFR mutations in CTCs in 84% of the patients corresponding to those present in matching tumor tissue. The majority of patients carrying sensitive EGFR mutations whose disease responds to drugs eventually develop resistance to these EGFR-TKIs. The T790M gatekeeper is a mutation of acquired resistance to TKIs. The development of third-generation EGFR inhibitors capable of overcoming T790M-associated resistance has led to a need for noninvasive methods of T790 detection to guide the selection of therapy[27]. A recent work comparing the T790M genotyping from tumor biopsies with analysis of simultaneously collected CTC and ctDNA, the resistance-associated mutation was detected in 47% to 50% of patients using each of the genotyping assay, with concordance among them ranging from 57% to 74%[28]. However, in those patients in whom the concurrent biopsy was negative or indeterminate, the two CTC and ct-DNA-based assays together enabled genotyping in 35% of these patients.

Detection and monitoring of NSCLC-specific rearrangements have also been reported. The diagnostic test for ALK rearrangement in NSCLC for crizotinib treatment has been evaluated by using a filtration enrichment technique and filter-adapted fluorescent in situ hybridization (FA-FISH). All ALK-positive patients had four or more ALK-rearranged CTCs per 1 mL of blood (median, nine CTCs per 1 mL; range, four to 34 CTCs per 1 mL). No or only one ALK-rearranged CTC (median, 1 per 1 mL; range, 0 to 1 per 1 mL) was detected in ALK-negative patients. This assay enabled both diagnostic testing and monitoring of crizotinib treatment[29].

Other crizotinib target is ROS1 rearrangement. These rearrangements have been evaluated in CTCs using ISET and FA-FISH in a small group of 4 patients whose tumor had these genetic aberrations. In this study, ROS1-gene alterations were detected in every patient analyzed. Tumor heterogeneity, assessed by ROS1 copy number, was significantly higher in baseline CTCs compared with tumor biopsies in those patients experiencing progression disease or stable disease[30]. Less common mutations have also been analyzed. BRAF is a rare oncogenic driver that is mutated in 2% of NSCLC and responds to BRAF inhibitors. A recent paper has reported on the feasibility of digital PCR to detect circulating BRAF-mutated DNA on both ctDNA and DNA extracted from CTCs in patients during targeted treatment for BRAF-V600E-mutated lung adenocarcinoma. These case series concluded that variations in BRAF-mutated ctDNA were correlated with a response according to RECIST criteria, with ctDNA being much more sensitive than CTCs[31].

In addition to the gain of prognostic information, CTCs as a biomarker are still not used in the routine clinical practice. Further validation in large multicenter studies to evaluate reproducibility, specificity and sensibility should be prior performed.

CIRCULATING FREE TUMOR DNA

Cell-free tumor DNA (cftDNA) exists in plasma or serum and is single- or double-stranded DNA. Most cell free DNA (cfDNA) is reportedly fragmented but is a potential tool to enable non-invasive diagnostic tests for personalized medicine in providing similar molecular information as the one derived from invasive tumor biopsies. Early studies showed that many cancer-associated molecular characteristics, such as single-nucleotide mutations, methylation changes and cancer-derived viral sequences, can be found in the cfDNA. These findings were significant for the development of ctDNA detection technology. Nevertheless, ctDNA-based assays are confronted with several challenges. Body cells release cfDNA into the bloodstream, but the majority of cfDNA is often not of cancerous origin, making it sometimes difficult to detect[12]. Also, prior knowledge about particular mutations is usually required, which may be hard to obtain. However, recent technological advances have overcome these restrictions, making it possible to identify both genetic and epigenetic alterations[32].

The fraction of cfDNA that is tumor derived in patients with cancer has a variable contribution ranging from < 0.1% to > 10% of the DNA, whose variability is thought to be affected by tumor burden, stage, cellular turnover, accessibility to circulation and factors affecting blood volume. The ctDNA thus released will load the same genetic (somatic) alterations as tumor cell and this genetic load may be detected and quantified. These platforms are real-time quantitative PCR (qPCR); digital PCR; Beads, Emulsion, Amplification and Magnetics (BEAMing); and Next-Generation Sequencing (NGS). The sensitivity ranges of these techniques range from 15% to 0.01%, but one of the weak points is the lack of standarization, in order to understand their clinical applicability[33].

For years, real time qPCR has been used for the identification of genetic alterations and nucleic acids quantification. While qPCR is a standardized, relatively inexpensive technique, its precision for quantifying rare allele or genetic alterations present at very low levels is significantly lower compared to other techniques[34]. However, most of the published studies adopted this technology for the analysis of ctDNA in lung cancer.

On the detection of the EGFR mutations in cfDNA using qPCR, Kimura et al. showed for the first time that plasma-derived EGFR genotype is predictive of subsequent clinical response to an EGFR-TKI. In the cases analyzed by both sequencing of the primary tumor and Scorpion ARMS assay of plasma, 72.7% of cases were concordant[35]. As well, Mok et al reported a concordance between tissue and blood tests of 88%, with blood test sensitivity of 75% and a specificity of 96% using the cobas blood test, based on qPCR[36]. EGFR oncogenic mutations have also been analyzed from plasma of patients included into several different trials[24,37]. The sensitivity of ctDNA in identifying EFGR mutations compared with the tissue ranged from 43 to 100%, being capable to identify EGFR mutations in patients with insufficient tissue.

The use of digital PCR has the potential for unparalleled precision enabling accurate measurements for low frequency genetic alterations quantification. dPCR allows for the detection of mutated cfDNA in a high background of wild type cfDNA and allows for small fold change differences to be detected. Several studies have assessed the feasibility of dPCR for biomarker testing and cancer monitoring and have evidenced that dPCR is indeed an adequate technologies for such purpose[38-40].

BEAMing is based on a combination of emulsion dPCR with magnetic beads and flow cytometry for the highly sensitive detection and quantification of mutant tumor DNA molecules[41]. BEAMing is a sensitive but complex method to detect known genetic mutations, specially when they are at a very low copy number[42]. This technology has been compared to mutation analysis of tumor tissue in a recent publication, testing 21 mutations in BRAF, EGFR, KRAS and PIK3CA with different cancers (13.8% of NSCLC). Results were concordant for archival tissue and plasma cfDNA in 915 cases for BRAF mutations, in 99% cases for EGFR mutations, in 83% cases for KRAS mutations and in 91% cases for PIK3CA mutations, demonstrating that BEAMing is feasible and highly concordant with tumor tissue analysis[43].

Taniguchi et al[3] used this technology for NSCLC patients to query for the evaluation of plasma testing for EGFR mutations in 44 patients with EGFR mutant NSCLC (23 with progressive disease after TKI and 21 TKI-naïve). Sensitizing mutations were detected in 72.7% of these patients, while T790M was detected in 43.5% of those with a

progressive disease.

To assess the ability of different technology platforms to detect EGFR mutations, including T790M, from ctDNA, a comparison of multiple platforms was undertaken[44], including two non-digital platforms (cobas ® EGFR Mutation Test and therascreenTM EGFR amplification refractory mutation system assay) and two digital platforms (Droplet Digital TM PCR and BEAMing digital PCR). For the T790M mutation, the digital platforms outperformed the non-digital platforms. Recent publication comparing also BEAMing and cobas test, showed a higher positive percent agreement between BEAMing plasma and tumor results than cobas EGFR plasma and tumor results (82% for activating mutations and 73% for T790M using BEAMing vs 73% and 64%, respectively, using cobas plasma test) [45].

The emerge of new drugs that target other genomic alterations rather than EGFR mutations have significantly increase the complexity of biomarker testing. In the context of an expanding number of driver mutations to test, NGS presents itself as a suitable technology since it allows simultaneous detection of multiple alterations in a very efficient manner. However, the use of this sequencing technology might be limited by a modest sensitivity. In this way, Uchida et al. reported a diagnostic sensitivity and specificity for exon 19 deletions of 50.9% and 98%, respectively; and for the L858R mutation of 51.9% and 94.1%, using deep sequencing as a detection system for EGFR mutation in ctDNA. As they analyzed patients of every stage, the overall sensitivity was 54.4% for all cases; stages IA-IIIA, 22.2%; and stages IIIB-IV, 72.7%, highlighting that NGS might be restricted to advance disease[46]. Similarly, Couraud et al used the IonTorrent Personal Genome Machine for the deep sequencing of the most relevant hotspot somatic mutations (EGFR, BRAF, KRAS, HER2, and PIK3CA) in tumor DNA (tDNA) and plasma cfDNA of never-smoker NSCLC patients. In ctDNA, 50 mutations (36 EGFR, 5 HER2, 4 KRAS, 3 BRAF, and 2 PIK3CA) were identified. Sensitivity of the test was 58% and the stimated specificity was 87%[47]. Because sensitivity appears to be compromised in early stages several strategies to solve this issue are under development. In this way, Newman et al. have developed an ultrasensitive method for quantifying ctDNA capable to detected ctDNA in every patient with stage II-IV NSCLC and in 50% of patients with stage I, with 96% specificity for mutant allele fractions down to 0.02%. Researchers communicated that ctDNA levels correlated with tumor volume and distinguished between residual disease and treatment-related imaging changes, and measurement of ctDNA levels allowed for earlier response assessment than radiographic approaches, facilitating personalized cancer therapy[48]. However, low quantities of cfDNA in the blood and sequencing artifacts could limit analytical sensitivity of this methodology.

To date, there have been communicated a high number of reports presenting the different clinical applications of ctDNA as liquid biopsy, specially, monitoring tumor burden, detecting resistance mechanisms, predicting response to treatment or detecting mutations in the absence of tissue[28]. Two meta-analysis have explored the diagnostic value of cfDNA for the detection of EGFR mutation status[49-50], both communicating a similar effectiveness (sensitivity of 67.4% and specificity of 93.5% vs sensitivity of 62% and specificity of 95.9%).

EXOSOMES

Exosomes have emerged as a novel mode of intercellular communication. Recent studies suggest that the secretion of functional mRNA and miRNA from one cell to another is, in part, mediated through exosome-mediated mechanisms[51].

Exosomes are involved in tumor initiation, growth, progression, metastasis, and drug resistance by transferring oncogenic proteins and nucleic acids. Therefore, exosomes and their load could be diagnostic, prognostic and predictive of responses biomarkers[52].

These vesicles of endocytic origin transfer information to the target cells, including proteins, DNA, mRNA, as well as non-coding RNAs, through at least three mechanisms: receptor-ligand interaction, fusion with the plasma membrane, or endocytosis by phagocytosis[53]. Thakur et al showed evidence that tumor-derived exosomes carry double-stranded DNA, showing that exosomal DNA represents the entire genome and could reflect the mutational

status of parental tumor cells[54].

Diverse technologies have been used for their isolation, as the magnetic activated cell sorting or ultracentrifugation, combined with sucrose gradient. Likewise, several methods, (e.g., transmission electron microscopy, Western blot, and FACS) have been used to characterize the isolated exosomes based on their biochemical (morphology, size or exosomal markers). However, there is a lack of the accurate method to determine the concentration of exosomes. The researchers have to rely on inaccurate measurements of protein concentration or nanoparticle tracking analysis. Quantitative RT-PCR, nucleic acid sequencing, Western blot, or ELISA have been used for exosome RNA and protein identification[55].

Circulating levels of tumor exosomes, exosomal small RNA and specific exosomal miRNA have been tested as diagnostic and prognostic markers in patients with adenocarcinoma[56].

A recent work[57] suggested that protein concentration of circulating exosomes and miRNA levels, using a 12 specific miRNA signature previously described to be elevated in lung cancer patients[58], are significantly higher in lung adenocarcinoma than in a control group.

The work also showed a similarity between the circulating exosomal miRNA and the tumor-derived miRNA patterns, suggesting that circulating exosomal miRNA might be useful as a screening test for lung adenocarcinoma. Similarly, another recent work reported a model based on microRNAs derived from circulating exosomes capable to discriminate between lung adenocarcinoma and granuloma[59], but further validation is needed to confirm the predictive power of this model.

Exosomal RNA appears to be a promising source for the identification of large rearrangements in liquid biopsies, such as the EML4-ALK translocation or ROS1 translocation, both being targets for therapy with crizotinib when found in cancer tissue.

Brinkmann et al have recently communicated the technical feasibility to detect EML4-ALK fusion transcripts in plasma samples from patients known to be positive by tissue FISH testing using exosomal RNA. The diagnostic test they explored, named ExoDx Lung (ALK) had a sensitivity of 88% and a specificity of 100%[60].

As exosomes are accessible in early all body fluids, urinary exosomes have been also screened as novel diagnostic indicators for NSCLC patients. Li et al. have detected a higher level of expression of leucine-rich α-2-glycoprotein (LRG1) in urinary exosomes by Western blot, further validated also in lung tissue by IHC, between NSCLC patients and healthy controls. These results suggested that LRG1 could be a candidate biomarker for non-invasive diagnosis of NSCLC in urine[61].

However, despite all the evidence, the exact mechanisms mediating the clear roles of exosomes in cancer have not yet been fully elucidated.

DISCUSSION

We believe that the monitoring of cancer through different liquid biopsy methodologies, together with correlation of more accurate imaging techniques (PET/CT and Radiology with functional CT and MRI), and adapting these to the new technologies already present in the routine clinical practice, will allow us to more precisely determine the response to treatment. Several studies[62-65] have revealed a statistically significant correlation between disease stage and the presence of tumor-associated genetic alterations in the blood of patients with several tumors. Currently, evaluation of response is only carried out as a standard procedure 3 months after initiating treatment, time by which the patient could be suffering side effects and toxicity without receiving any benefit, and without the opportunity of changing treatment. In this respect, lung cancer presents an added difficulty, namely the limited amount of tumor that can be obtained for molecular studies. Given the difficulty, the cost and the increased workload to obtain the sample, acquisition of tumor DNA is one of the limiting factors for patient selection. The availability of

non-invasive tests that can detect molecular alterations in different genes will allow the study of biomarkers in all patients, thus overcoming these limitations and thereby radically changing clinical practice and increasing the treatment options for our patients.

Over the last decade, lung cancer treatment has evolved from administration of chemotherapy yielding poor outcomes, to biomarker assessments, such as EGFR, ALK or ROS1, with remarked improvements in OS[66]. So, time has changed and personalized medicine has become a reality. However, to fully enable personalized medicine it s desirable to have an easily accessible, minimally invasive way to follow the molecular profile of a patient's tumor longitudinally. The peripheral blood of a cancer patient is a pool of cells or cell products derived from the primary tumor or from the metastatic sites. The analysis of these blood components provides a mean for minimally invasive molecular diagnostics, overcoming limitations of tissue acquisition, which is not always possible and satisfactory in NSCLC patients.

Technical developments achieved during the last years allow the identification and characterization of CTCs, cfDNA, exosomes and, recently, tumor-educated platelets, all known to play a main role in the carcinogenesis, relapse and progression of cancer. However, some of these analytical platforms typically have a suboptimal sensitivity[67].

On the other hand, PET/CT and SUV assessments are widely criticized for being insufficient and imprecise, however to date no methods for reanalysis have been developed that also interact with tumor molecular status. Moreover, the combined, parallel histological study of tumor biopsies, with data regarding regression to treatment and which attempts to investigate cisplatin resistance mechanisms, may contribute to the design and use of new drugs. The liquid biopsy study using different techniques and procedures, and their correlation with histological response, the PET images and functional radiological studies could prove as a valuable tool for the development of future clinical trials.

At a time when increasingly more new drugs are appearing, and with the unravelling of more complex molecular mechanisms, it is ever more necessary to translate these advances to the patient's bedside.

However, such advances are of little use if the chosen target population is not homogeneous, or is insufficiently or incorrectly assessed, or the research is so far removed from the patient that it will probably never contribute to improving a health problem.

Several studies, some of them analyzed in this editorial, have tried to focus on its role for screening and early detection of cancer, estimation of risk for metastatic relapse, identification of therapeutic targets and resistance mechanisms, and real-time monitoring of patients[68]. As cancer is known a complex and dynamic disease, future prospective trials are needed to demonstrate its clinical utility. All these different blood products might contribute to an improved survival of cancer patients.

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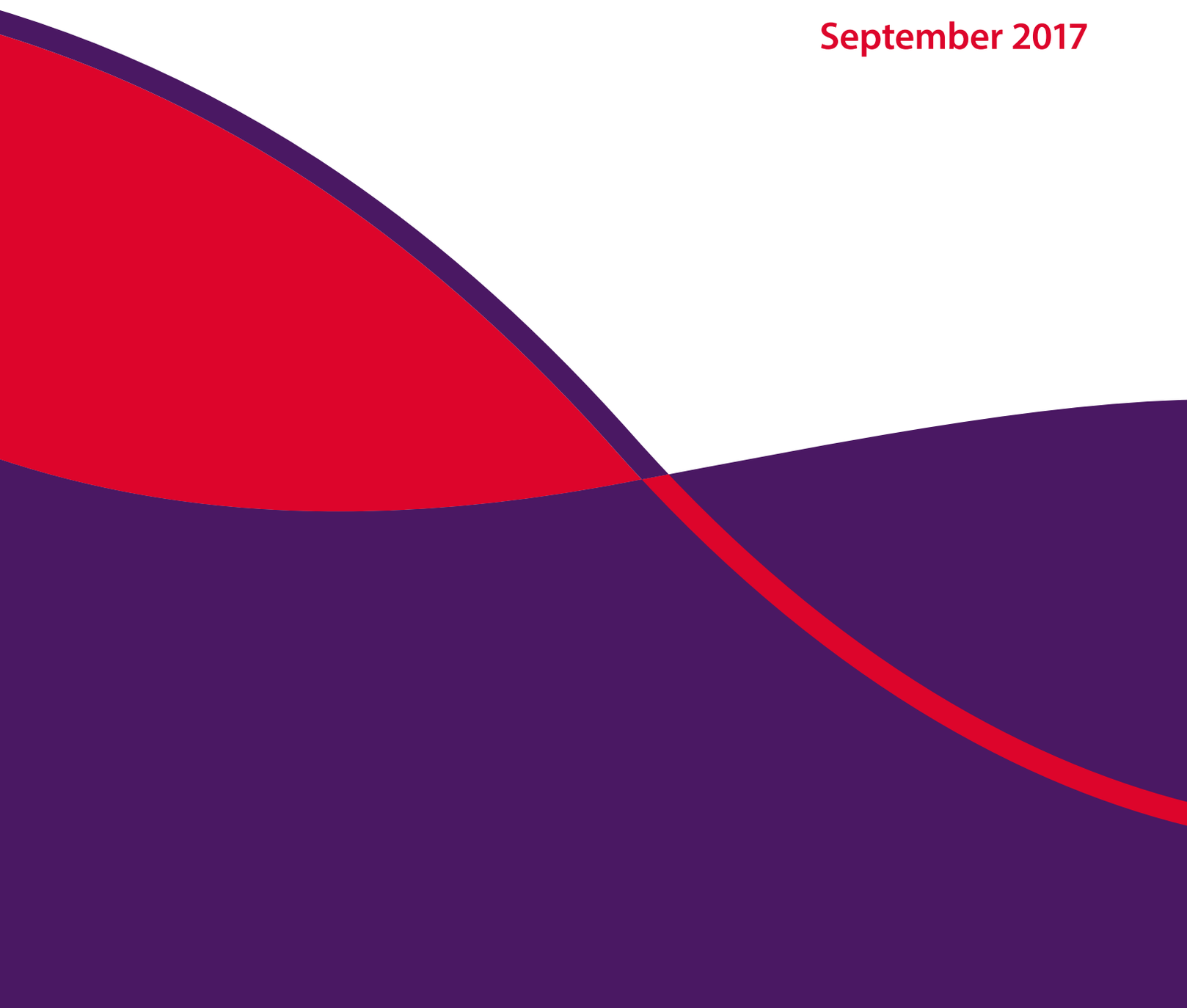
Developing effective ctDNA testing services for lung cancer

September 2017



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NB: URLs in this report were correct as at 31 July

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1. Executive summary

Personalised medicine – a revolution in cancer care?

The era of personalised medicine is upon us, but amid all the promise, and the hype, what does this mean for patients? For patients with cancer, personalised medicine can mean monitoring or clinical intervention based on family history or evidence of inherited susceptibility mutations, or it could mean a therapy that targets tumours with specific genetic mutations. For a sub-set of patients with non-small cell lung cancer (NSCLC) a group of drugs called tyrosine kinase inhibitors (TKIs) treat tumours with mutations in the *EGFR* gene. These patients require a genetic test in order for the clinician to prescribe the therapy. Due to the challenges associated with carrying out a biopsy to collect a solid tumour sample for testing, many patients miss out on genetic testing. In patients for whom biopsies are possible approximately 30% of biopsies fail or do not yield enough material for a genetic test.

Circulating tumour (ct) DNA testing – a pioneering technology to meet clinical need

A new genomic technology, circulating tumour DNA testing, can bridge the gap for these patients. ctDNA testing analyses patient blood samples for mutations in the fragments of tumour DNA found in the circulation. This non-invasive biopsy method has advantages in terms of accessibility and ease of use, allowing patients who would not have received a genetic test from a solid biopsy the chance to have a test carried out using another method.

While there are still technological challenges to overcome in terms of the use of ctDNA technologies, the evidence from clinical trials, combined with the availability of targeted therapies, has been sufficient to see the adoption of ctDNA testing within a small number of NHS laboratories in the UK. Testing is currently used at diagnosis, to determine if a patient's tumour has mutations in *EGFR*, and at progression, to determine if resistance to therapy is caused by an additional mutation in *EGFR* called p.T790M, for which a second line TKI is available.

ctDNA testing is already benefiting some patients with NSCLC; however, this technology is not in widespread use throughout the NHS and not all eligible patients receive testing. The question is how this technology might be delivered in the most effective and equitable way across the health system to ensure that every eligible NSCLC patient receives a ctDNA test as appropriate to their care.

How can the health system maximise the impact of ctDNA testing for NSCLC patients?

A multidisciplinary workshop was held on 7th March 2017 to investigate the current position of NHS ctDNA services for NSCLC patients, with participation from key experts including NHS clinical scientists, clinicians and the commercial sector.

The discussions at the workshop informed the content of this report, which describes the early experiences of some of the laboratories that have pioneered the introduction of this testing into the NHS. It outlines the steps that stakeholders, both internal and external to the health system, will need to take to maximise the impact of this technology for lung cancer patients. Finally, details are provided on emerging possibilities to applying this technology across the care pathways for the management of advanced lung cancer and other cancers.

The workshop also focused on what needs to be done to strengthen and support implementation, improve quality of testing and ensure that more patients receive testing. The key findings are that:

The NHS should consider offering ctDNA testing of *EGFR* in lung cancer to all eligible patients. The evidence of clinical utility demonstrates that ctDNA testing makes a difference to patients, increasing accessibility to targeted therapies. While some improvements are needed to laboratory techniques, these are not a barrier to adoption, since the alternative is that patients miss out on testing, and therefore some will not receive appropriate targeted therapy, which has been assessed as cost-effective treatment for NHS use.

The NHS should use existing ctDNA testing to maximise access to TKIs. To do this, we recommend that the health system support existing services – ctDNA testing at diagnosis and for p.T790M – and establish current services as centres of excellence for ctDNA testing in lung cancer. Clinical guidelines from NICE, or developed by the clinical community, are crucial for raising awareness and providing reassurance to clinicians that these tests have clinical utility and benefit patients. Ongoing NHS service evaluations will provide critical evidence to support wider NHS implementation.

There are lessons that can be learned from existing ctDNA services that will support the wider use of ctDNA tests in future. The laboratory case studies presented demonstrate that there are different paths to take in terms of test development and delivery, which are affected by the resources and infrastructure available in each laboratory. The meeting highlighted the importance of ongoing external quality assurance efforts, which will provide benchmarks on the quality of testing services. Active engagement efforts to inform the health system about ctDNA testing are effective, as demonstrated by the example of the All Wales Medical Genetics Service, but require the laboratories to invest time and resource. Further efforts such as these will be required, however, to improve further engagement within the health system.

Recommendations to support the development of effective ctDNA testing services

Evidence from early adopters of ctDNA technology revealed that whilst testing benefits NSCLC patients in terms of accessing the most appropriate treatment, there are issues that need to be addressed in order to support the implementation of comprehensive, effective and equitable ctDNA testing for NSCLC. Based on the findings, we make seven recommendations to meet these challenges. Establishing a solid foundation now in terms of service development and delivery will be an investment for the future, when more uses of this technology are likely to become available.

- Healthcare commissioners should formally consider the provision of ctDNA services in lung cancer and improve and strengthen current service provision
- Ongoing service evaluation is required to ensure that the health system has the appropriate information for further implementation
- Laboratory websites should include up-to-date and clear electronic referral information and resources, including testing information, costs and logistics
- Engagement about ctDNA testing can take place within the multidisciplinary team (MDT) – ideally via an individual who can act as a point of contact for queries and information. This person could be a clinician, clinical scientist or a pathologist
- Clinical guidelines on the use of ctDNA testing in NSCLC should be developed
- Service establishment and validation should be supported, by and within the health system, by promotion of available funding, promotion of test funding structures, linking of test development into accelerated access of technologies and support of collaborative test development
- NHS England should consider how patients can have improved access to funded targeted therapies and take steps through policy development to ensure that the health system is better prepared to implement targeted therapies when commissioned

2. Personalised medicine in cancer

Cancer care is changing. Over the past 15 years or so there has been an increase in what is termed personalised medicine, whereby interventions are targeted at specific groups of patients based on genetic, physiological or other clinical factors. In cancer care, a personalised therapy does not just refer to a drug that targets a tumour with a particular genetic make-up, but also to targeted screening based on family history or other risk factors, preventive interventions based on genetic risk, or therapeutic approaches that take into account a patient's predicted prognosis.

These targeted approaches can help to bring higher-risk patients into the treatment pathway sooner, sparing them more invasive treatment later on, or aim to benefit patients by prescribing treatments that are more specific to their needs and increase overall- or progression-free survival with fewer side effects.

While targeted approaches to cancer care have contributed to much improved breast cancer survival rates (see Box 1), other cancers, such as those affecting the lung, have not benefited to the same extent.

Box 1. Targeted approaches to breast cancer

The breast cancer drug Herceptin, one of the first-approved targeted cancer drugs, is prescribed to women with *HER2* positive cancer and has improved overall and progression-free survival compared to routine chemotherapy. Also in breast cancer, the OncotypeDx panel test examines the activity of 16 breast cancer associated genes in women with ER+/Her2- breast cancer and those with a low risk score can be spared chemotherapy and its associated side-effects, receiving hormone therapy only. Greater cancer awareness and targeted approaches such as earlier detection, improved surgery, radiotherapy and chemotherapy, have raised the breast cancer survival rate from 4 in 10 in the 1970s to 8 in 10 today.

2.1. Lung cancer – area of unmet clinical need

Lung cancer is the second most common cancer in the UK, after breast cancer, with nearly 47,000 cases diagnosed and around 36,000 deaths per year[1]. Despite progress made in diagnostics, surgical techniques, drug regimens and radiotherapy, only 5% of lung cancer patients survive for 10 years or more post-diagnosis, a figure that has remained relatively constant for the past 40 years.

There are a variety of reasons for this, the main ones being:

- **Late diagnosis**: 75% of patients are diagnosed with stage III or stage IV cancer – that is, cancer that has advanced from its original site into the surrounding tissue, or has metastasised (spread) to other parts of the body
- **Comorbidities:** these present a challenge when treating this patient population. Smoking is implicated in nearly 90% of lung cancer cases, which can cause additional health problems such as chronic obstructive pulmonary disease (COPD) and cardiovascular disease. Taking these comorbidities into consideration when treating lung cancer patients will have an impact on treatment selection

It is clear that a range of clinical approaches will be needed to improve lung cancer survival. Improved strategies for early detection will play a role, but also incremental improvements in and access to the treatments that clinicians already have at their disposal.

Diagnosing and characterising lung cancer

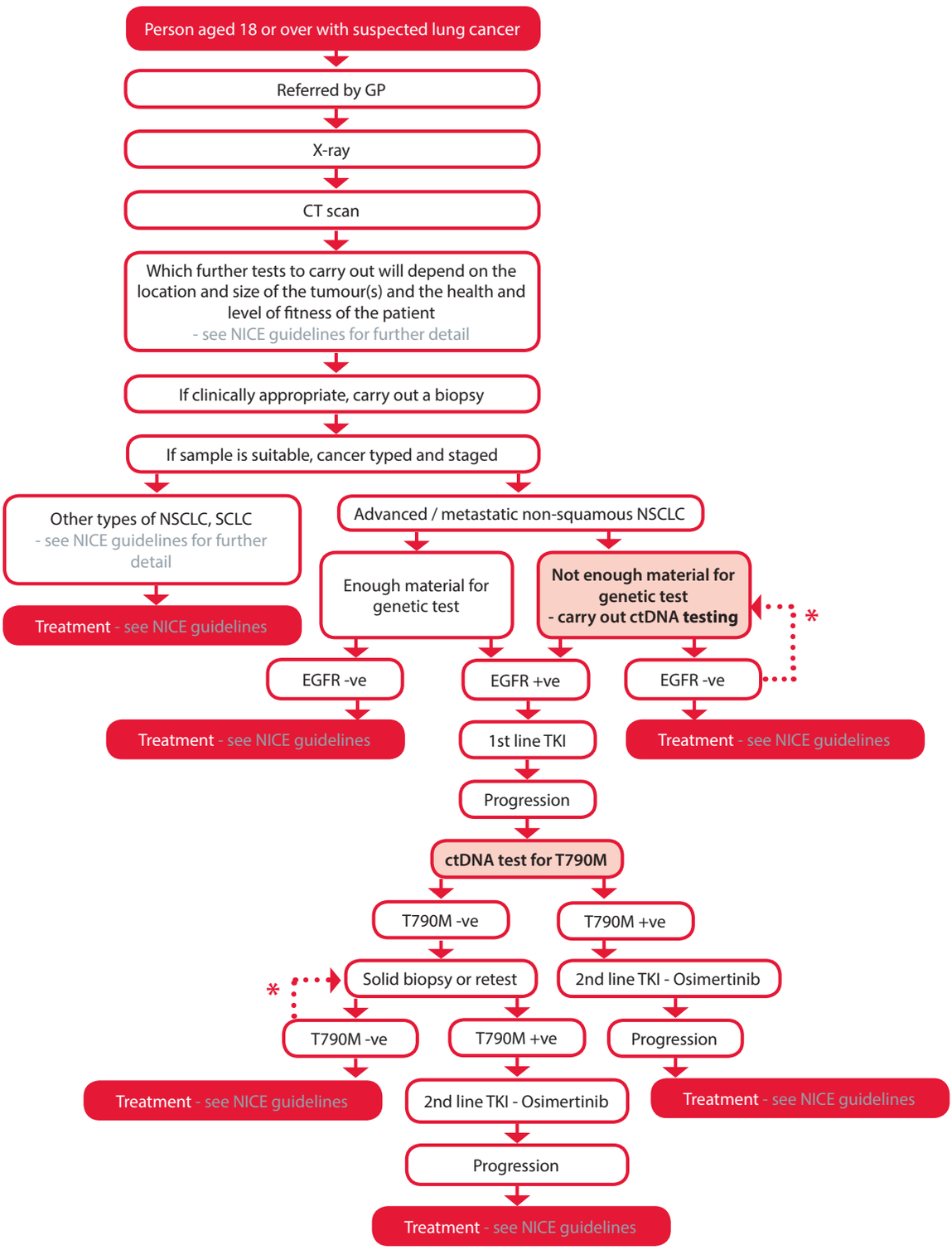
Patients with suspected lung cancer symptoms are referred by their GP for an X-ray (Figure 1). If lung cancer is suspected after the X-ray, the patient is referred to a respiratory physician. The patient will have a CT scan to more accurately determine the location of lesions and the results are used to decide what further tests might be needed to accurately diagnose and stage the cancer. What tests are carried out will depend on:

- Location of tumour(s) in the chest and possibly outside the chest
- Size of the tumour(s)
- Health and level of fitness of the patient

A tumour sample is needed for accurate staging and diagnosis. Easily accessible and small tumours are usually surgically resected – most commonly in patients with stage I or II cancer (see Table 1). Alternatively a sample can be taken using a CT-guided needle biopsy through the chest wall, or a bronchial biopsy if a tumour is more centrally located in the lung. If the tumour site is unsuitable or inaccessible for biopsy or the patient cannot tolerate a biopsy for health reasons, then sputum cytology will be attempted.

If a suitable sample is collected through any of these methods, the pathology laboratory will prepare samples – solid tumour samples will be fixed in formalin and embedded in a paraffin block (FFPE). The type (Figure 2) and stage of the cancer will be determined by examining the characteristics of the cells in the sample. If the tumour is of a type potentially suitable for targeted treatments, then further biochemical or genetic tests will be carried out on the sample. The clinical team will use this information to plan treatment. While FFPE blocks have historically been an effective way of preserving and storing tumour samples, this procedure can affect the quantity and quality of DNA extracted from the tissue sample. This has a major impact on successful whole genome sequencing (WGS) and as such, the 100,000 Genomes Project is implementing pathways to collect fresh frozen tumour samples to preserve DNA for genomic testing, and also working on improved 'genomic friendly' tissue handling protocols to provide high quality DNA samples from FFPE tissue samples.

Figure 1. Example lung cancer patient pathway showing where ctDNA testing could be used



This pathway is based on the comprehensive pathways for lung cancer available from NICE (pathways.nice.org.uk/pathways/lung-cancer) amended to show where ctDNA testing could have an impact on patient care.

*Testing could be repeated at this stage however guidelines are required on the use of repeat ctDNA testing (see page 45).

Challenge in diagnosis - biopsy failure

Biposy failure is one barrier to accurately subtyping lung cancer. In some patients, due to their state of health or due to tumour location, it is not possible to collect a tissue sample. In other patients, a biopsy is carried out but there is only enough tissue for a diagnosis and not enough tissue for further biochemical or genetic tests that could help the clinical team to personalise treatment. These issues mean that approximately 30% of biopsies are classified as 'failed', restricting patient access to the tests needed to determine if targeted therapies are suitable for their cancer.

Figure 2. Subtypes of lung cancer[1, 2]

87%

Non-small cell lung cancer

Adenocarcinoma, which develops from the cells that make mucus in the lining of the airways and is the most common type of primary lung cancer. It often develops in outer parts of the lung and grows more slowly than other types of lung cancer. It is the most common type of lung cancer diagnosed in non-smokers (approximately 40% total lung cancer cases)

Squamous cell cancer, which develops from the epithelial cells that line the airways often in a central position and near a main bronchus (airway), and is often caused by smoking (25-30% total lung cancer cases)

Large cell carcinoma, a quick-growing cancer so-called because the cells look large and rounded under a microscope and which can appear in any part of the lung (10-15% total lung cancer cases)

12%

Small cell lung cancer

Usually caused by smoking and rarely develops in someone who has never smoked

>1%

Rare carcinoid tumours

Start in hormone producing cells

Table 1: Summary of lung cancer disease stage, treatment and prognosis

Disease stage*	Non-small cell lung cancer	Small cell lung cancer
I and II 20.3% cases	Thermal ablation, radiotherapy. Surgery is the gold standard	Radiotherapy, surgery
Prognosis	40–70% survive 5 years (stage I) 25–45% survive 5 years (stage II)	20–40% survive 5 years (stage I and II)
III 18.6% cases	Multimodality treatment. Surgery, chemotherapy, some radiotherapy	No targeted treatment. Chemotherapy and brain radiotherapy
Prognosis	~20% survive 5 years	10–15% survive 5 years
IV 47.3% cases	Chemotherapy, targeted therapy, radiotherapy, supportive care. Palliative care	Chemotherapy: etoposide and platinum. Prophylactic brain radiotherapy
Prognosis	Chemotherapy: 6–12 months Median Overall Survival (MOS) Targeted therapy (TKIs): 2 year MOS; 2–13% survive 5 years	Chemotherapy: 6–12 months MOS; radiotherapy or supportive care can add 2 months. 1% survive 5 years

*13.8% cases are 'stage not known'.
Reference for incidence and survival statistics: www.cancerresearchuk.org/about-cancer/type/lung-cancer/treatment/statistics-and-outlook-for-lung-cancer

Targeted treatments for lung cancer

Non-small cell lung cancer (NSCLC) is a genetically diverse (heterogeneous) disease. Several mutations have been discovered that drive tumour growth. Drugs that target some of these driver mutations have been available on the NHS since 2010 (Table 2). These drugs all demonstrate improved progression-free survival and overall survival compared with standard of care chemotherapy.

Targeted therapies currently available on the NHS target mutations affecting two genes:

- *ALK* – anaplastic lymphoma kinase gene. In 3-5% of NSCLC an *ALK-EML4* fusion drives cancer growth. This fusion is detected using fluorescence *in situ* hybridisation where tumour cells are stained with a specific dye and examined under the microscope
- *EGFR* – epidermal growth factor receptor gene. A number of mutations in *EGFR* are known to drive tumour growth i.e. substitutions, deletions and insertions in exons 18-21. *EGFR* mutations are found in 10-15% of NSCLC patients in European populations. They are more common in females, those of East Asian ethnic origin and in light smokers or those who have never smoked. A genetic test on tumour tissue is required to detect *EGFR* mutations – these are mostly found in non-squamous adenocarcinomas[3] therefore all patients with this type of cancer should be tested

Table 2: Targeted lung cancer drugs currently available on the NHS in England

Drug	Targeted mutation
Crizotinib (Xalkori), ceritinib (Zykadia)	*ALK* fusion – blocks overactive anaplastic lymphoma kinase. Ceritinib is an option for patients whose cancers have stopped responding to crizotinib
Geftitinib (Iressa), erlotinib (Tarceva) First generation tyrosine kinase inhibitors (TKIs)	Target first line mutations in *EGFR*
Afatinib (Giotrif) Second generation TKI	Target first line mutations in *EGFR*
Osimertinib (Tagrisso) Third generation TKI	Targets cancers previously treated with 1st/2nd generation TKIs that have developed resistance due to the p.T790M mutation in *EGFR*

Why prescribe a TKI?

Patients with *EGFR*-positive NSCLC have a better response rate on first line tyrosine kinase inhibitors TKIs, (targeted lung cancer drugs) than on standard of care chemotherapy, while *EGFR*-negative patients have a worse response on TKIs compared to chemotherapy[4]. *EGFR*-positive NSCLC patients taking TKIs have a median overall survival of two years, which is an improvement on 6-12 months overall survival for these patients on chemotherapy. TKI drugs have further advantages over chemotherapy: they are taken as a tablet, once a day, so patients do not need to come into hospital for treatment and they have fewer side effects.

2.2 ctDNA testing - improving access to targeted therapies

As *EGFR* mutation negative patients do worse on TKIs than on chemotherapy it is not possible for a clinician to prescribe TKIs without a genetic test. Therefore it is vital that clinical teams, and patients, have access to accurate and reliable genetic testing.

However, as outlined above, a significant proportion of tumour biopsies fail. A recently developed and validated technology, circulating tumour (ct) DNA testing, often known as liquid biopsy, can be used as an alternative to solid tumour biopsy testing and can increase patient accessibility to *EGFR* genetic testing and TKI treatment.

Clinicians require a genetic test on a tumour sample in order to prescribe TKIs (targeted lung cancer treatment). ctDNA testing gives an alternative to solid tumour biopsy testing and increases patient access to *EGFR* testing and TKI treatment

This form of testing is not currently available to test for *ALK* fusions. While ctDNA analysis has been used to detect *ALK* fusions in patient studies, it is technically more challenging and yet not ready for use in clinical practice[5].

What is cell free (cf) and circulating tumour (ct) DNA?

It has been known since the 1940s that fragments of cell free (cf) nucleic acids can be found in the bloodstream of healthy individuals[6]. This DNA comes from healthy cells in the body, mostly haematopoietic (blood and immune) cells found in circulation or in the bone marrow, that have died due to the body's natural process of programmed cell death (apoptosis)[7]. cfDNA levels in the blood also change in specific clinical circumstances – in pregnant women cfDNA of fetal origin (cffDNA) can be detected in the maternal bloodstream and DNA from tumours can be detected in the bloodstream of cancer patients (Box 2).

The first application of testing to detect and characterise cfDNA in clinical practice occurred in fetal medicine. Cell-free fetal DNA was first identified in maternal plasma in 1997[8] and is detectable from around eight weeks gestation; at around 21 weeks gestation, the percentage of cfDNA in the mother's blood stream that comes from the foetus has risen to around 10%, increasing steadily thereafter as the pregnancy progresses[9]. Non-invasive prenatal testing (NIPT) for aneuploidies (e.g. Down's syndrome) was first shown in 2007[10] and NIPT is now available in many countries, including on the NHS from 2018[11], demonstrating that testing to detect cfDNA is deliverable in a clinical setting.

Researchers first reported the presence of raised cfDNA in the serum of patients with cancer in 1977[12], suggesting that DNA from tumours also makes its way into the circulation. Tumour cells release fragments of DNA, typically 160-180 base pairs in length, into the patient's bloodstream via a variety of mechanisms, such as secretion, apoptosis or necrosis (cell death resulting from injury or damage)[13]. Circulating tumour (ct) DNA differs from cfDNA in that it reflects the genetic makeup of the tumour and as such is an alternative source of tumour genetic material to that extracted from solid tumour samples. Tests to detect ctDNA took longer to develop than those for cffDNA since the total percentage of DNA in the blood that comes from a tumour and contains a detectable mutation can be much lower – down to 0.01%, or fewer than 10 mutant DNA fragments per ml blood[14] – meaning that more sensitive methods needed to be developed to reliably detect it.

Box 2. Types of cell free circulating DNA

Cell free DNA (cfDNA) - DNA from healthy cells in the body, found in the bloodstream

Cell free fetal DNA (cffDNA) - DNA from the foetus (specifically cells in the placenta) found in the bloodstream of pregnant women, in addition to maternal cfDNA. Typically over 10% of the total DNA in circulation during pregnancy

Circulating tumour DNA (ctDNA) - DNA from tumours found in the bloodstream of cancer patients, in addition to cfDNA from the patient's healthy cells. Typically 0.01-10% of the total DNA in circulation

2.3. Uses of ctDNA testing in medicine

Circulating tumour DNA testing (liquid biopsy) is an alternative method to solid tumour testing for monitoring the common mutations of tumours. There are a range of possible uses of ctDNA tests in clinical care, which include:

- **Diagnosis or early detection**

  ctDNA tests could potentially be used in screening programmes to detect early disease in asymptomatic patients or to diagnose those with suspected cancer

- **Treatment selection**

  The availability of therapies that target tumours with particular characteristics means accurate tests are needed to determine which therapies are suitable for which patients. Liquid biopsies can be used in situations when a solid tumour sample is not available

ctDNA testing can also be used in clinical scenarios where solid tumour biopsy testing is impractical or clinically challenging, if not impossible:

- **Monitoring response to treatment**

  Once treatment has started, regular ctDNA testing could be used to monitor treatment response. It has been shown that changes in ctDNA levels in patients who have been monitored during treatment correlate with their tumours' response to treatment[14, 15]. For patients on targeted therapies, ctDNA can be monitored for emergence of resistance mutations

- **Monitoring relapse after treatment with curative intent**

  Once a patient has had surgery or other treatment with curative intent, there is always the question of whether any residual disease remains. ctDNA tests can be used to monitor patients to detect relapse or if all of the tumour was removed by the initial treatment

Extensive progress has been made in the development of ctDNA tests which are now being used in clinical practice to inform treatment decisions. Many technical and biological challenges remain, however, which are discussed in chapter 5.

3. ctDNA testing in lung cancer in the NHS

ctDNA testing services for lung cancer are being delivered by a small number of NHS laboratories in the UK. While the development of these services is a positive advance, they are not being used to their full potential and a proportion of eligible patients are not receiving testing. This report considers three key questions / challenges that must be addressed if the NHS is to capitalise on the work of these early pioneering services to harness the benefits of ctDNA testing technology for patients:

- Should the NHS offer ctDNA testing of *EGFR* in lung cancer to all eligible patients?
- How should the NHS use ctDNA testing to maximise access to TKIs?
- What lessons can be learned from existing ctDNA services to support the wider use of ctDNA tests in the future?

3.1. Drivers for the development of ctDNA testing services in the UK

For patients with NSCLC, ctDNA tests are a viable alternative for determining *EGFR* mutation status. There are several points along the patient pathway where ctDNA testing could be used to inform treatment decisions (see Figure 1) and a number of factors have contributed to the development of ctDNA testing services in NHS laboratories:

1. **The development of targeted therapies that were approved by NICE for use in lung cancer patients** – gefitinib (2010), erlotinib (2012), afatinib (2014), osimertinib (2016, on the Cancer Drugs Fund in England) (see also Table 2). The development of osimertinib coincided with the development of more sensitive and specific ctDNA tests and the drug information states that plasma or solid tumour testing can be used as a companion diagnostic. In addition, the product information for gefitinib was updated in 2014 to include ctDNA testing as a viable companion diagnostic test. The information for osimertinib also states that plasma or tissue are suitable samples for testing. The information for erlotinib and afatanib state that an *EGFR* mutation test should be carried out, using '*well validated and robust methodology*' – test method or sample type are not specified
2. **Area of unmet clinical need** – patients not having access to tests due to solid biopsy failure (not enough material for a genetic test), or because a biopsy cannot be carried out either for clinical reasons or because the tumour is inaccessible
3. **Advances in pre-analytical and analytical performance of ctDNA tests** – these include increased sensitivity, specificity, reduced cost, and ease of use
4. **Laboratories motivated to launch services** – supported by clinician demand and pharma support

Advantages of liquid biopsy over solid biopsy

- **Minimally invasive** – only needs a blood sample, which can be taken during a regular appointment and does not require specialist staff to carry out. The test can also be easily repeated if necessary
- **Improves accessibility to genetic testing and to targeted therapy**
- **Better captures tumour genetic diversity (heterogeneity)** - particularly if a patient has tumours in multiple sites
- **Cheaper than biopsy -** a biopsy is CT guided and performed by a radiologist. For cost comparison, a ctDNA test costs £170 and a biopsy £1,000 without complications/associated care. However this pathway is unlikely to be cost saving in terms of the overall cost of treating the patient, since liquid biopsy is often used after a failed solid biopsy, as outlined above. Health economic analyses are required to resolve this question
- **Fewer side effects** - blood test avoids the side effects of solid biopsy and potential hospital admission to deal with solid biopsy complications.

What is the current guidance on EGFR *mutation testing in NSCLC?*

NICE guidance

The NICE guidance on lung cancer[16] and the technology appraisals (TA) for TKIs both state that TKIs can be prescribed once a successful genetic test on the tumour has taken place (TA310, TA374, TA192, TA258, TA227, TA416[17]). The guidance refers users to the *EGFR*-TKI diagnostics guidance, DG9[18], for information on how to carry out *EGFR* mutation testing. These state that '… DNA extraction and mutation analysis can be carried out on the biopsy tissue..' and that 'if biopsy tissue is not available, DNA extracted from cytology samples can be used for mutation analysis. Other molecular tests may be performed as clinically indicated.'

In January 2017 a four week consultation took place on transferring DG9 to the 'static guidance' list. In their reply to consultation responses, many of which included comments on plasma testing for *EGFR*, the committee stated that it 'is aware' that plasma samples can be used for ctDNA testing and stated that the Qiagen Therascreen kit and Roche cobas® tests are being considered as subjects of future Medtech innovation briefings.

The TAs for first line TKIs do not specifically state the correct medium for a test, only that a test should take place before a prescription decision is made. In the TA for osimertinib (second line TKI), the cost-benefit analysis provided in the background information includes ctDNA testing, meaning that ctDNA testing could be used to test for the p.T790M mutation, however this is not explicitly stated and is likely to be causing confusion among users.

The Scottish Intercollegiate Guidance Network (SIGN)

The SIGN guidelines for lung cancer (2014) currently only include information on tissue testing for *EGFR*[19]. However, the Scottish Medicines Consortium advice on osimertinib states that: 'Molecular pathology experts advise that eligible patients would be identified using a plasma-based circulating tumour DNA (ctDNA) test. This would be followed by a tissue test (biopsy) in patients with a negative result from ctDNA due to the possibility of false negative results'[20].

The All Wales Medicines Strategy Group (AWMSG)

The AWMSG information on osimertinib says 'Product meets AWMSG exclusion criteria due to NICE appraisal'[21]. Osimertinib is available on the Cancer Drugs Fund (CDF) in England but the CDF is not available in Wales, so patients can access osimertinib via Individual Patient Funding Requests, which fund treatments not routinely provided by NHS Wales.

The Royal College of Pathologists

The Royal College of Pathologists has guidance in the form of datasets for histopathology reporting on cancers and the dataset for lung cancer recommends steps that pathologists can take when handling solid tumour specimens to ensure that enough tissue is left over for a genetic test should one be required. While they do not address the issue of plasma testing, they do recommend, in light of different working practices at different hospitals, that local guidelines are put in place to determine who orders genetic tests[22]. There is therefore an opportunity for guideline development on plasma testing at a local level via the MDT.

International guidelines

The latest international guidelines, from College of American Pathologists, International Association for the Study of Lung Cancer, Association for Molecular Pathology (2013)[23] and a European Working Group (2015)[24] do not address the issue of plasma testing for *EGFR*. Australian recommendations for *EGFR* p.T790M testing in advanced NSCLC state that a plasma sample can be used for testing, followed by a solid biopsy if the test is negative or a repeat liquid biopsy after six weeks[25].

3.2. ctDNA test methods

Measuring levels of ctDNA does present challenges, although advances in sample preparation and sequencing technology are making these easier to manage.

ctDNA forms a small proportion of the total DNA in the circulation, the majority being DNA from healthy cells. This proportion can vary greatly, from less than 0.01%, which pushes the limit of detection of the most sensitive methods at our disposal, to up to 40%[14, 15, 26]. This range is due to factors such as tumour size – for many cancers, more advanced and/or bigger tumours are associated with higher levels of ctDNA[27]. The levels of cf- and ctDNA in the blood can also vary due to time of day, exercise, trauma or infection[28, 29]. Each of these characteristics are not yet fully understood but it is clear from measurements made in patients that ctDNA levels can vary greatly from person to person. Even in advanced disease, the amount of ctDNA present varies greatly between individuals and in some ctDNA cannot be detected, for reasons that are not yet fully understood [27]. These factors can have an effect on the usefulness of a ctDNA test for some patients, but are not a barrier to the use of current ctDNA testing in lung cancer. However, if the potential of ctDNA testing is to be fully exploited, more research into the biology of ctDNA and its release into the bloodstream is needed.

ctDNA has a short half-life, estimated to be approximately between 15 minutes to 2.5 hours[28]. One study estimated a ctDNA half-life of 114 mins in a patient who had surgery to remove their tumour[14]. Until relatively recently this meant that blood samples had to be processed almost immediately after collection. However the development of blood collection tubes containing preservative mean that samples can now be stored for several days at room temperature before analysis, if the sample has been collected correctly.

Options for test development

A range of molecular assay methods can be used to analyse ctDNA (Table 3) and there are a variety of factors that help to determine which path laboratories take to deliver a clinical service – either by developing their own method, or by validating a CE-marked or US Food and Drug Administration approved 'out of the box test' such as the Roche cobas® or Qiagen Therascreen. These factors are:

- **Sensitivity and specificity of the method (Table 4)**
- **Time and infrastructure** – does the laboratory have enough time and/or infrastructural capacity to develop its own test? If not, validating an 'out of the box' test might be a more appropriate choice as it is less time-consuming. The manufacturer develops the test protocol and validating laboratories must ensure that the test is performing to the manufacturer's specifications
- **Does the laboratory have equipment and/or experience in using a technique in another context** e.g. in clinical research? If yes, the laboratory can take advantage of expertise already developed by repurposing existing equipment and methods. Developing their own test is more research and time intensive but does mean the laboratory has complete control over every step of the process and can optimise test development for its specific needs

Table 3: Summary of ctDNA analysis methods

Technique	Limit of detection	Optimal application
Sanger sequencing	>10%	Tumour tissue
Pyrosequencing	10%	Tumour tissue
COLD-PCR and pyro	2%	Tumour tissue
NGS	2%	Tumour tissue
Q-PCR	1%	Tumour tissue
ARMS	0.1%	Tumour tissue
Roche cobas®, Qiagen Therascreen (adapted for ctDNA)	0.1%	ctDNA
ddPCR, BEAMing	0.01%	ctDNA

Adapted from *Liquid biopsies: genotyping circulating tumor DNA*[30]. Limit of detection determines the lower limit of the percentage of mutant DNA that can be detected as a fraction of the cfDNA extracted from the sample.

Table 4: Sensitivity and specificity of selected ctDNA methods for *EGFR* mutations, compared to tissue as a reference standard

Test	Mutation tested	Sensitivity	Specificity	Concordance	Reference
Roche cobas®	Panel of *EGFR* mutations	75%	100%	96%	[31]
Roche cobas®	*EGFR* exon 19 deletion p.L858R mutation p.T790M mutation	82% 87% 73%	97% 97% 67%	- - -	[32]
ddPCR	*EGFR* p.L858R mutation	90%	100%	97%	[32]
ddPCR	p.T790M	71%	83%	74%	[32]
ddPCR	*EGFR* p.L858R mutation	80-82%	95-98%	94%	[33]
BEAMing PCR	p.T790M	81%	58%	-	[32]
Therascreen	Panel of *EGFR* mutations	73%	99%	95%	[31]

All laboratories are striving to provide the best test and external quality assurance (EQA) efforts, currently ongoing, will provide quality benchmarks for testing and provide greater clarity as to which is the best testing method to use in a clinical setting (section 5.2). In the meantime, laboratories have taken a pragmatic approach to accelerate patient access to testing by optimising test development according to available resources and infrastructure. While this may mean relying on a less than maximally sensitive but already validated technique, these efforts mean that laboratories are in a position to offer patients testing and to respond to technology improvements and the results of EQA as and when they arise.

Two commonly used methods in UK laboratories are:

- **The cobas® diagnostics platform**, developed by Roche, provides diagnostic tests for a range of medical conditions. The *EGFR* mutation test v2 identifies 42 mutations in exons 18-21 of the *EGFR* gene and can be used on plasma or tissue samples, giving a result in around four hours. Sample preparation kits are available for both tissue and plasma samples as well as the cobas® z 480 system, a device that prepares samples for PCR and carries out the PCR reaction. Finally the cobas® system provides automated result interpretation and test reporting. This type of system is useful for laboratories that do not have the capacity to develop their own test – everything required is provided and the laboratory only needs to validate the technology to confirm that it is working to the manufacturer's specifications. The disadvantages, according to a study comparing different ctDNA detection technologies, are that digital and BEAMing PCR are more sensitive and better able to detect p.T790M than Roche cobas® or Qiagen Therascreen[32]. The technology is also less flexible, for example laboratories cannot analyse mutations not covered by the test, since any interference with the mutation panel invalidates the test's IVD (*in vitro* diagnostic) status.

- **Digital droplet (dd)PCR** is a technological advance on traditional PCR. The sample under investigation is divided into many smaller samples, for example microwell plates or oil emulsion droplets. An individual PCR reaction takes place in each well or droplet. By dividing the sample up in this way, rare molecules in the sample are more likely to be detected. The number of positive droplets can be counted and thus also gives a measurement of the amount of target DNA in a sample. The advantages of using ddPCR is that it is an accurate and sensitive method and a good choice for the detection of low-concentration ctDNA molecules. The development and use of a ddPCR assay requires a certain level of expertise and requires equipment that is not necessarily standard in most molecular pathology laboratories. This is a time-consuming and resource intensive process for a laboratory. However once established ddPCR techniques are accurate and flexible – for example, new mutations can be added for investigation.

3.3. Why should ctDNA testing be used in lung cancer care in the NHS?

Increased interest and research activity in ctDNA technologies have resulted in the development of ever more sensitive methods that can detect a variety of mutations via a liquid biopsy. The approval of ctDNA tests by the US Food and Drug Administration (FDA) and the European Medicines Agency (CE-marked) have contributed to advancing the use of ctDNA testing in clinical practice and in NSCLC the benefits of ctDNA testing include reduced risks to the patient, ease of sample collection and reduced turn-around times[28, 34] (see also laboratory turnaround times in chapter 4).

While there is more work to be done on the biology of ctDNA and on technology improvement, research studies and clinical trials have demonstrated that ctDNA is an accurate proxy of mutations within a tumour[31, 35]. These studies have also examined the effectiveness of TKIs versus standard of care in eligible patients and the difference in lung cancer patient outcomes in those given a solid tumour test against those given a liquid biopsy test[36].

For example, the ASSESS clinical trial evaluated the 'real-world' performance of ctDNA testing compared to tissue based testing. It showed that mutations were less likely to be picked up by plasma tests due to lower sensitivity of ctDNA testing[31]. However, results from the AURA3 trial showed that patients with positive p.T790M tests did equally well in terms of progression free survival when prescribed osimertinib regardless of whether they had had a plasma or a solid tumour test[36].

Continuing technological improvements in ctDNA tests should improve the lower sensitivity of liquid biopsy compared to solid biopsy. However test sensitivity should also be considered in the context of access: the development of liquid biopsy is improving patient access to genetic testing and therefore to targeted therapy if their tumour has a targetable mutation.

In the UK approximately 12,000 lung cancer patients per year have the type of cancer eligible for a genetic test. Assuming a 30-40% biopsy failure rate, ctDNA testing is now an option for up to 4,800 patients who would otherwise not have had their eligibility for TKI therapy assessed. For patients who have already been prescribed TKIs (approximately 1,800) and who progress on treatment, a ctDNA test is now considered the first line test to determine if the p.T790M resistance mutation is present in the patient's tumour and thus whether the patient is eligible for osimertinib therapy. Clinicians will only consider a solid biopsy to test for p.T790M if the ctDNA test is negative. This reduces the number of patients having another invasive and more expensive solid tumour biopsy, since patients who have a positive p.T790M ctDNA result are spared another biopsy.

In the UK approximately 12,000 lung cancer patients per year have the type of cancer eligible for a genetic test. Assuming a 30-40% biopsy failure rate, ctDNA testing is now an option for up to 4,800 patients who would otherwise not have had their eligibility for TKI therapy assessed

There is currently a significant range of evidence from clinical trials to demonstrate that patients benefit from ctDNA testing in lung cancer. As such the laboratories currently offering testing have assessed the potential benefits to patients as being sufficient to make the investment in service development. While some refinement to techniques is necessary, for example to improve test sensitivity compared to solid tumour testing, ctDNA testing works in patients in whom a result from a solid biopsy is not possible. Formal evaluation of these tests is still needed, however, for example to inform the development of guidelines.

Benefits of offering ctDNA testing of *EGFR* to NSCLC patients

- More patients receive clinically appropriate TKIs
- Improved clinical outcomes. Patients who otherwise might not have been prescribed TKIs can receive them and as a result have improved outcomes in terms of progression-free survival

4. Pioneering ctDNA test provision on the NHS

ctDNA testing for patients with NSCLC is available as an NHS service from a small number of laboratories in England and Wales, either as fully accredited services or as services under development. These laboratories have invested time and resource in developing and validating testing services, working with their clinical colleagues to develop tests in the routine clinical care of NSCLC patients. However testing is not currently available routinely to all NSCLC patients and we need to learn from the experiences of early adopters of this technology if we are to efficiently and equitably expand services.

4.1. How is ctDNA testing being used?

There are currently two situations where ctDNA testing is being used in clinical practice in the UK:

- At diagnosis, when insufficient or no tissue is available for a genetic test a plasma ctDNA test is performed to determine *EGFR* mutation status and whether first line TKIs can be prescribed
- At progression, when a patient taking first line TKIs has clinically progressed a ctDNA test is carried out to determine if the p.T790M resistance mutation is present. If the test fails or is negative, a solid biopsy can be attempted

Here we present cases studies from three of these early adopters outlining how services can be established and what drivers and barriers each laboratory encountered.

The laboratories featured below took a variety of approaches to test development, each making use of a distinct combination of technical capabilities, prior research experience and capacity for in-house test development. We outline early results in terms of initial service provision and what key lessons emerge from these laboratories' experiences.

Table 5: Routine NHS ctDNA testing services

Location	Approximate population coverage
Validated testing services	
All Wales Medical Genetics Service	3 million, also offer services to SW England, potentially another 5.3 million
University Hospitals Birmingham – Queen Elizabeth Hospital	1.1 million
Manchester Centre for Genomic Medicine	2.8 million, up to 5 million in NW England
Royal Marsden/Institute of Cancer Research	4 million, but receives referrals from elsewhere, particularly specialist cases
Leeds Genetics Laboratory, The Leeds Teaching Hospitals NHS Trust. p.T790M only at present	Approximately 4 million
Northern Ireland Molecular Pathology Laboratory (NI-MPL)	1.8 million (population of Northern Ireland)
Royal Surrey Hospital / Berkshire and Surrey Pathology Services	2 million (Surrey and Berkshire)
Under development	
Sheffield Children's NHS Foundation Trust/ Sheffield Diagnostics Service. p.T790M test validated to launch soon, in process of validating cfDNA NGS panel assay	Around 1 million
Clatterbridge Cancer Centre in Liverpool/ Royal Liverpool Hospital	Around 2.5 million in Merseyside and Cheshire
Molecular Malignancy Laboratory, Addenbrooke's Hospital, Cambridge	Approximately 4-5 million in East Anglia
Edinburgh and Lothians Laboratory Medicine. Offering ctDNA testing for p.T790M but service is not currently ISO accredited	Approximately 1.5 million in the region (Scottish population 5.4 million)
Newgene, Newcastle. Working up clinical assay for *EGFR* mutation analysis	Approximately 3 million in North East England and North Cumbria

All the laboratories offer testing to patients within their catchment but also from outside – in theory any NHS clinician who chooses to can send samples for testing to one of these centres.

Case study: Manchester Centre for Genomic Medicine

The Manchester Centre for Genomic Medicine is a leading centre for clinical genomics, employing around 250 staff to deliver genetic testing and counselling to patients. It delivers clinical genetics services (adult and paediatric), biochemical and genetic testing for metabolic disorders, cytogenetics, molecular pathology, specialist cell culture services and bioinformatics services. The Centre also undertakes clinical research and takes part in clinical trials in collaboration with the University of Manchester and other stakeholders. The Centre covers a catchment area of up to 5 million people in North West (NW) England but receives referrals from across the UK – requests for ctDNA testing have been received from Scotland, Newcastle, NW England and London.

Drivers of service provision

The ctDNA service evolved from existing research and clinical trials protocols – the laboratory works closely with the Christie Hospital and was involved in clinical trials research using ctDNA. As such, early technique development was supported by these projects. The drivers for service development came from clinical oncologists and the availability of drugs requiring companion diagnostics.

One of the challenges of setting up the service is that it coincided with a period of intense technology development by companies. When new technologies emerged onto the market, the laboratory assessed their effectiveness relative to the techniques that they were already using. The laboratory was therefore able to implement technological improvements as they became available, however this did require a significant time and investment concentrated in a short period of time. The laboratory was able to absorb test development into their available resources, participation in AstraZeneca-funded clinical trials allowed them to develop testing techniques in this context.

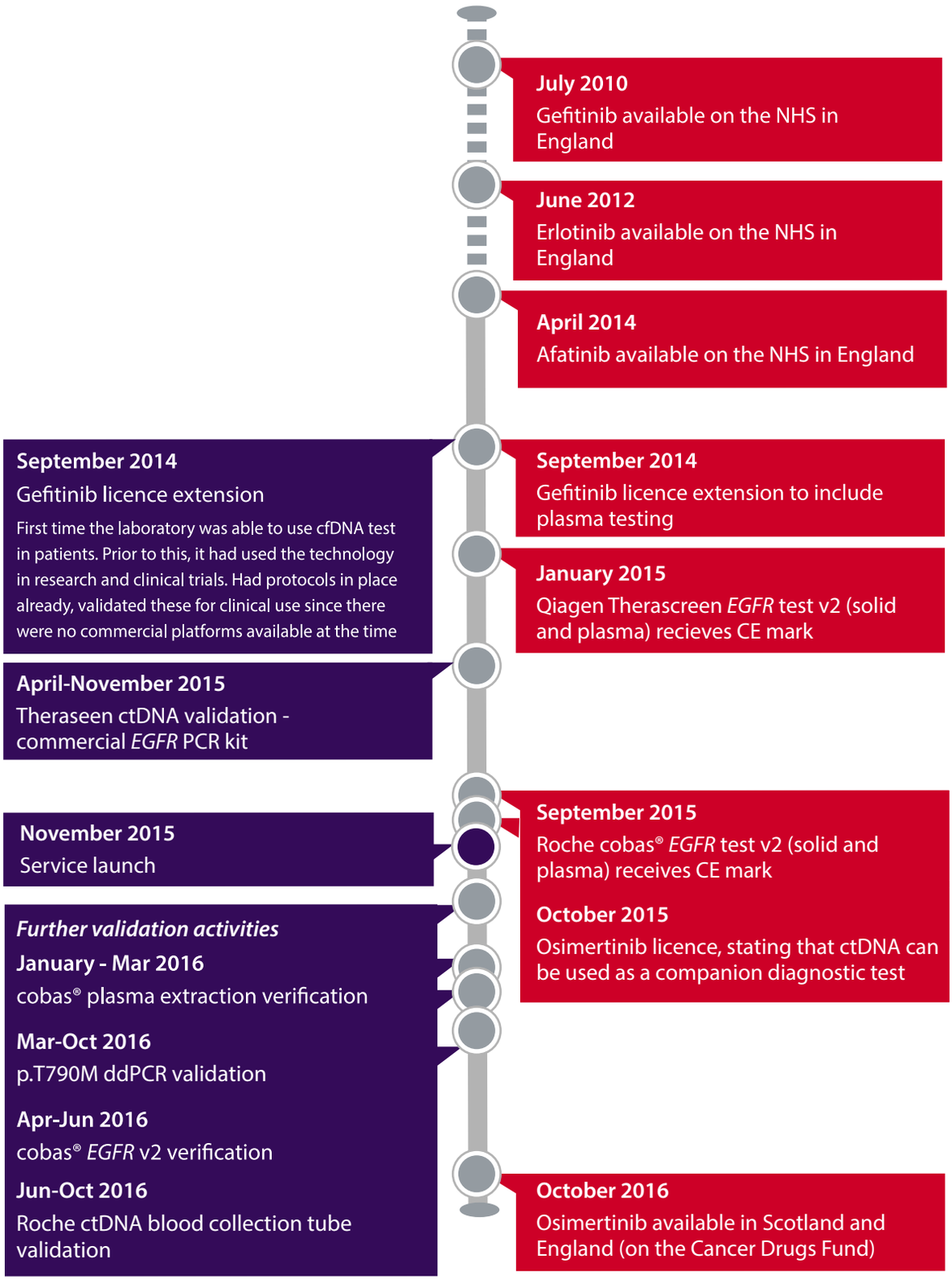
Current service

The current workload for the laboratory is approximately six tests per week for first-line TKI screens and three per week for p.T790M mutations. Plasma tests have a quicker turnaround time from sample collection to test result since blood samples, unlike solid samples, are not sent to the pathology laboratory first before being sent for genetic testing. The turnaround time for plasma is 3.3 working days compared to 5.5 for tissue.

Table 6: Summary of cases analysed and results since service launch (up to March 2017)

	No. samples	Positivity rate
Screens (plasma at primary diagnosis)	101 (16 Therascreen, 85 cobas®)	9.9% (this compares to 11.5% in tissue)
p.T790M	73 (47 samples, 64%, had adequate ctDNA)	34% (in adequate samples). Detection rate depressed by early Therascreen tests and improved following inclusion of ddPCR

Timeline for service development



Case study: University Hospitals Birmingham NHS Foundation Trust – Queen Elizabeth Hospital

The laboratories at Queen Elizabeth Hospital Birmingham (QEHB) offer biochemistry, cellular pathology, haematology, microbiology and transfusion services to University Hospitals Birmingham and external sites, covering a population of approximately 1.1 million people in the greater Birmingham area, extending to 5.7 million in the West Midlands.

The Molecular Pathology Diagnostic Service (MPDS) at QEHB is one of the largest solid tumour genetic-profiling centres in Europe providing a repertoire of tests that are available to both internal and external users. The laboratory has extensive experience in molecular testing of lung cancer and processes more than 300 *EGFR* tissue tests a month, using the validated Roche cobas® platform.

The laboratory first offered early access plasma testing in April 2016 with clinical service launch in October 2016.

Drivers of service provision

Several factors contributed to the laboratory developing a ctDNA testing service:

- The expectation of licencing allowing the testing of ctDNA to allow access to 3rd line TKI (osimertinib)
- Availability of a CE-IVD marked kit, Roche cobas®, for testing
- Complement the current solid tumour testing for *EGFR* performed in the laboratory
- The availability of stabilising tubes improving the logistics around getting blood samples to the laboratory

The Roche cobas® was selected for ctDNA testing as the technology was already available in the laboratory and was already being used for solid tumour EGFR testing. Therefore there were no capital costs involved and the technology was already familiar to laboratory staff. It also allowed the concurrent running of tumour and plasma samples thus offering an efficient testing strategy and removing the need for batching. Both solid and plasma tests cover all of the clinically relevant mutations within *EGFR*. Furthermore, cobas® was the assay used in the trial in which the laboratory had participated so they already had experience of delivering testing in a research context. Test development and service delivery occured using available resources – no additional members of staff were required.

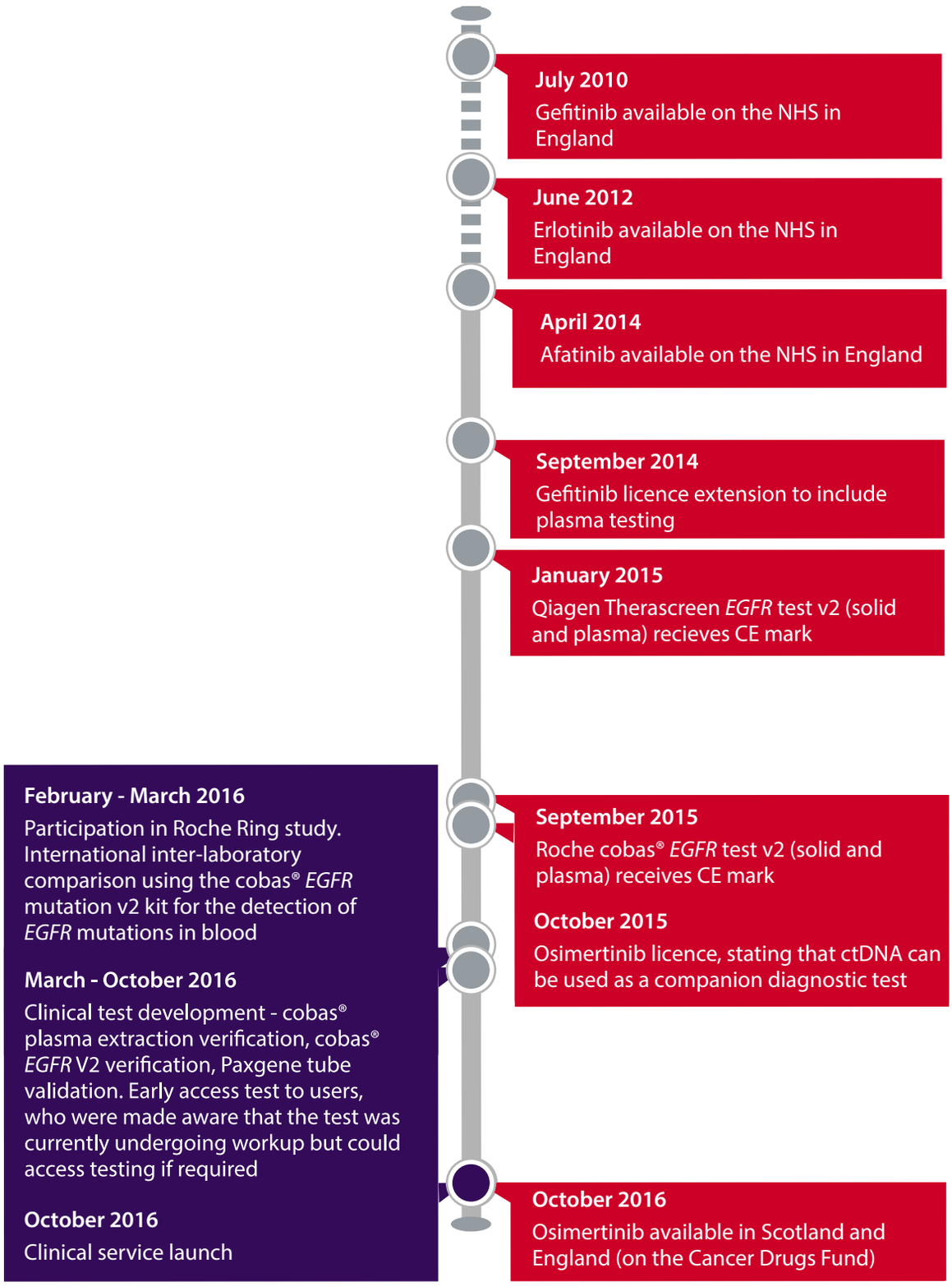
Current service

So far the laboratory has received 308 cases. Inadequate clinical data upon receipt of samples means that classification of some samples was problematic as staging was not available. The data in the table reflects where clinical details were sufficient to categorise the sample. In addition ten patients are undergoing monthly monitoring (range of two to seven repeats received).

Table 7: Summary of cases analysed and results since service launch (up to June 2017)

	No. samples	Positivity rate
Screens (plasma at primary diagnosis)	12	2 (16.5%)
p.T790M	185 (total) of which 104 (original mutation detected)	40 (18.4%) 40 (33.6%), where original mutation detected and hence an indicator that ctDNA level adequate

Timeline for service development



Case study: All Wales Medical Genetics Service

The All Wales Medical Genetics Service (AWMGS) based at the University Hospital of Wales, Cardiff, provides constitutional genetics services, and cancer genetics (solid and haemato-oncology). The service sits within the Directorate of Laboratory Medicine which collectively offers services in cytology, haematology, histopathology, biochemistry and immunology, microbiology and phlebotomy.

The AWMGS provides clinical genetics services for the population of Wales but will also carry out ctDNA testing requests from English hospitals.

Drivers of service provision

The ctDNA testing service was developed over a number of years and relied on a range of individuals – trainees, clinician researchers and clinical scientists – to carry out the work necessary to develop and validate techniques. As outlined below, some of this work was possible due to external funding of staff positions. The laboratory chose ddPCR as its preferred method of analysis due to previous experience of other technologies and the sensitivity of the ddPCR method.

Current service

The patients selected for the clinical validation had been chosen based on a confirmed *EGFR* sensitising mutation result on biopsy within the laboratory. Patients were referred from the Velindre Cancer Centre, Cardiff.

Currently the laboratory sends blood preservative tubes to clinicians when they want to carry out a test. Plasma is stored and batched and there is a ctDNA run twice a week. The number of tests per week fluctuates from roughly two to six samples. Turn around time is less than five working days. The laboratory has received 137 samples from hospitals in Wales and in the south west of England.

Table 8: Summary of number of samples tested and results (as of June 2017)

	Screens (plasma at primary diagnosis)	p.T790M	Total
Within Wales	48	39	87
Outside Wales	13	37	50
Total	61	76	137
Positivity rate	13.11%	22.37%	

Timeline for service development

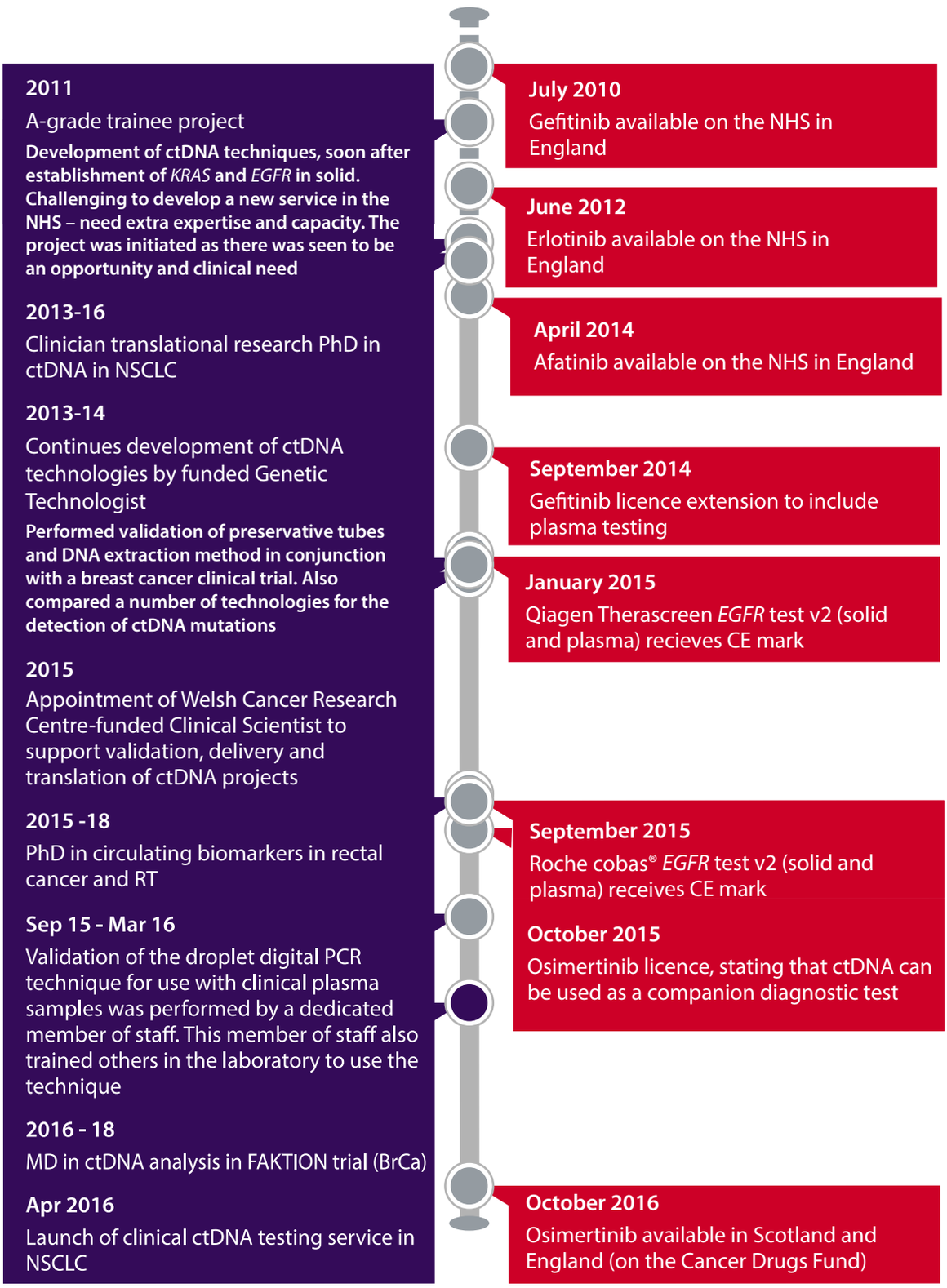


Table 9: Summary of laboratories' ctDNA testing implementation

	Manchester	Birmingham	Cardiff
Collection tubes	Roche cfDNA BCTs	Paxgene	Streck or CellSave
ctDNA extraction method	Roche cfDNA extraction	Roche cfDNA extraction	QIAamp (Qiagen)
Assay method: *EGFR* mutation detection (first line)	cobas® v2 mutation detection (42 *EGFR* mutations)	cobas® v2 mutation detection (42 *EGFR* mutations)	ddPCR
Assay method: p.T790M detection	cobas® v2 mutation detection plus ddPCR	cobas® v2 mutation detection (42 *EGFR* mutations)	ddPCR

4.2. Laboratory accreditation and test validation

All laboratories that deliver medical genetic testing in the UK must be accredited to the ISO15189 standard or be in the process of transitioning to this standard. Accreditation provides assurance to users that a service has been independently evaluated against recognised standards. When implementing new tests, accredited laboratories undergo a process of test validation, an essential and rigorous process to ensure that diagnostic tests meet standards and are fit for purpose, i.e. that when used in the laboratory the test is repeatable, reliable and robust, addresses the clinical question in hand, and has no impact on patient safety. ISO15189 clause 5.5.2 states that: '*The laboratory shall use only validated procedures for confirming that the examination procedures are suitable for intended use*'. While generic guidance is available on validation, laboratories have to develop their own internal guidelines and there is a high degree of professional judgement involved in validating a test. There is a balance to be struck between the accuracy, sensitivity and specificity of methods and ease of use – for example, a laboratory might accept a slightly lower yield for a DNA extraction method if the method is easier to use.

During the process of validating ctDNA tests, laboratories highlighted the importance of excellent communication and collaboration with their clinical colleagues to obtain the patient samples needed. In the case of ctDNA tests, patient blood samples are required before their treatment has started in order to accurately compare the performance of the plasma test with the solid tumour test. Support from clinical teams ensured that samples could be collected from patients at the appropriate point in their clinical journey.

Lessons from current ctDNA service provision

Factors contributing to the successful implementation of ctDNA services:

- **Collaborative working with clinicians to develop services and processes**, driven by clinician demand for services
- **Each laboratory took an approach that worked for them and suited their way of working and expertise** – using the methods that best suited them. This highlights an unanswered question – is there an optimal way of delivering a testing service and is there a best test?

However, laboratories bore the financial and clinical risk. They invested time, money and expertise in developing these services, either absorbing the costs internally or by making use of different external sources of funding. While the laboratories above were able to validate new tests without compromising existing service delivery, financial, infrastructural or logistical constraints can make this a challenge for other laboratories. This could have an impact on future implementation of ctDNA testing services if provision expands when new tests become available.

5. How can the health system maximise the utility of emerging ctDNA services?

Research studies and the development and validation of accredited services by laboratories demonstrate that ctDNA testing can deliver accessible genetic tests to patients with NSCLC in whom solid tumour testing is not possible. The question now is, how can the health system make the most of the pioneering services already established to ensure equitable access to all patients? What are the issues that are having an impact on the ability of laboratories to deliver services and to appropriately expand the reach of services to more patients nationally? What are the outstanding clinical research questions that need to be addressed and what are the areas where the clinical community, the health system and external organisations can work together to optimise the use of current services and build the evidence base, laying stable foundations for future service provision?

5.1. Clinical utility

While clinical trial results and initial results from the laboratories offering testing indicate that ctDNA testing in NSCLC is clinically useful and improves patient access to targeted therapies, there are unresolved questions surrounding the clinical scenarios in which testing can be used and areas for technical improvements.

What are the opportunities for future service improvements?

The ctDNA tests currently used are fit for use in the NHS, however there are a number of opportunities to improve their performance either through technical enhancements or resolution of underlying questions about the biology of ctDNA. Some of these are highlighted below.

Is there a best test for specific clinical circumstances?

Clinical trials have included analyses of different test methods used however this has been in the research context[31]. Ongoing external quality assurance efforts (see section 5.2) will help to address the question of which methods work best in a clinical context, as opposed to in a research context. The question can also be framed in terms of the expertise and resources available in the laboratory, i.e. the best test is that which the clinical laboratory has expertise in using and has validated. However if EQA efforts demonstrate that a test falls below established benchmarks in sensitivity and specificity then laboratories using that test should validate the EQA 'best test'.

Concerns over test sensitivity

Although ctDNA tests are less sensitive than solid tumour tests, this should be considered in the context of how ctDNA testing is providing information for drug treatment – as a second line test, in a situation where a first line test has failed or cannot be attempted. In this situation, lower test sensitivity can be an acceptable compromise compared to not carrying out a test at all. At this stage the key to addressing concerns will be ensuring that clear information is available to clinicians about the limitations of the test, but also how it can benefit patients. These issues are discussed further in section 5.3.

Table 10: What are the implications of reduced ctDNA test sensitivity on test results?

Test result	What does this result mean?	Outcome for the patient
True positive	Patient has *EGFR* mutation, which the test detects	Receives TKI therapy. Improved progression free survival compared to chemotherapy
True negative	Patient does not have *EGFR* mutation, which the test confirms	Receives chemotherapy
False positive	Patient does not have *EGFR* mutation, however test is positive for *EGFR* mutation (specificity issue)	Negative for patient – patients negative for *EGFR* mutation have a worse outcome on TKIs. Want to minimise/avoid this outcome
False negative	Patient does have *EGFR* mutation, however test is negative (sensitivity issue). Could be due to technical test failure or patient not releasing much ctDNA into the blood	Loss of potential benefit to patient as they do not receive TKIs. However will receive chemotherapy. Patient could also be retested if they progress on chemotherapy

In terms of test improvement, the goal with *EGFR* ctDNA testing should be to minimise the false positive rate i.e. optimise specificity even if the trade-off is reduced sensitivity. While false negative results are not ideal for patients, this outcome is not actively harmful.

How to report negative results

ctDNA testing is a new technology and as such there are still some uncertainties over how to distinguish true negative results from false negative results. This presents a challenge for reporting negative results to clinicians. The onus is on laboratories and clinicians familiar with the technology to report back negatives in an understandable way, clearly outlining the implications of a negative result and the options available, if any. Currently, the processes and guidelines are not clear but can be developed over time as service providers and users learn from using testing in a clinical setting. Initially it is appropriate to take a conservative approach to manage negative results – currently, if a patient is being tested for the p.T790M mutation, it is common practice to carry out a solid tumour biopsy if the liquid biopsy is negative and if the patient is a suitable candidate e.g. they are well enough to tolerate the procedure and their tumour(s) is accessible. A strategy employed by the laboratories offering testing is to also re-test patients for their original *EGFR* mutation at the same time as testing for p.T790M, if there is enough DNA: if the original mutation is detected but the p.T790M mutation is not, then this builds confidence in the negative result to p.T790M. An alternative method could be to carry out a repeat test after a set period of time. However, clear guidelines are needed to set out how often and when serial testing can take place. For example, Australian guidelines recommend that p.T790M retesting is an option, after a period of six weeks[25].

Clear guidelines are needed to set out how often and when serial ctDNA testing can take place. For example, Australian guidelines recommend that *T790M* retesting is an option, after a period of six weeks

When to test

Levels of circulating DNA differ according to time of day, exercise levels, obesity or illness, however these need to be better characterised. For patients with cancer, there is still much to learn in terms of how ctDNA levels change during disease progression, for example:

- Is there a better time of day to sample?
- Are there patient or clinical characteristics that correlate with more successful ctDNA tests?
- Is it useful to test before there is clinical or radiological progression, and change treatment accordingly?

It is not yet clear at what point the appearance of ctDNA in the blood indicates a clinically relevant change in a tumour. Clinical trials that use serial testing could help resolve this issue by investigating more deeply the interaction between ctDNA levels, progression of disease and treatment success. For example, the TracerX study used ctDNA testing to profile the evolution of early-stage NSCLC and to monitor 24 patients after surgery. Using ctDNA testing the team identified 90% of the patients who went on to relapse, up to a year before clinical progression of disease was detected by imaging[37]. As clinicians and laboratories grow in experience in delivering and using these tests, they will be able to develop guidelines that help to answer some of the questions raised about when to test.

Is solid tumour testing the gold standard?

When a patient has advanced or metastatic cancer, tumours are located in different sites in the chest and other parts of the body. In addition, only a subset of cancer cells will express first line *EGFR* mutations, and of these cells only a further subset will have p.T790M. Therefore if you want to carry out a solid tumour biopsy, which site do you choose? For p.T790M testing on progression, it is possible that a plasma test better reflects the clinical situation inside the body, reflecting both inter- and intra-tumour heterogeneity[38]. The AURA3 trial showed that there was no difference in response rate or progression free survival in patients treated with osimertinib who had their p.T790M status determined by plasma or solid tumour testing[36]. It can be argued that liquid biopsy is the gold standard for p.T790M testing – all laboratories that offer testing carry out a liquid biopsy first, only proceeding to a solid biopsy if the test is negative. This approach is also recommended by the Scottish Medicines Consortium guidance on osimertinib[19]. Ongoing evaluation of the testing services and clinical outcomes will be necessary for the optimisation of services and to enable wider implementation. An evaluation involving the laboratories at Cardiff, Manchester, Royal Marsden, Birmingham and Belfast is due to report results in early 2018.

5.2. External Quality Assurance

Early approaches to service development have been diverse, varying according to the expertise and resources in each laboratory. The molecular pathology community has recognised, however, the pressing need to undertake comparative benchmarking of the different approaches and to converge towards establishing best practice for ctDNA testing. EQA is the principal process through which this can be achieved. By determining which processes/tests perform the best, EQA provides benchmarks of quality that can stimulate improvements in test provision and motivate laboratories to match the best that is available. It also provides assurances as to the quality and standards of the tests available on the NHS.

The International Quality Network for Pathology, IQNPath, is an organisation that facilitates the exchange of information and expertise and brings together external quality assurance organisations in their efforts to implement new or improved testing methods in clinical practice.

A pilot EQA was set up via IQNPath which aims to:

1. Investigate the feasibility of delivering a technically challenging EQA
2. Assess the ability of laboratories to detect ctDNA in plasma samples
3. Assess the standard of reporting ctDNA testing results
4. Observe any differences between extraction methodologies and testing method strategies
5. Promote high quality ctDNA testing through guidelines

One of the challenges of the pilot project was how to mimic a standard set of blood samples for each laboratory, since it is not possible to send a representative patient sample to each laboratory. Two companies prepared artificial DNA samples which were spiked into pooled plasma samples and distributed to the participating laboratories. Seven European expert laboratories took part in the validation phase of the pilot, using a range of sequencing methods – Therascreen, cobas®, BEAMing, ddPCR, NGS – and extraction methods. The process, completed by March 2017, validated the artificial plasma samples, showing that the reference materials work, and a number of logistical issues such as transport of samples were resolved.

Phase two of this pilot EQA project is underway – validated artificial plasma were distributed to 32 international laboratories in March 2017.

5.3. Education, awareness and outreach

While the initial phase of service development involves a close working relationship between a laboratory and a group of clinicians who are usually located in the same hospital, once testing has been validated it is necessary to increase awareness and engagement of services to ensure that they are being fully utilised and that all clinicians have the opportunity to use testing for the benefit of their patients.

The All Wales Medical Genetics Service (AWMGS) undertook a formal programme of engagement between scientists and clinicians to raise awareness of the ctDNA testing service. When the service was being validated, the laboratory needed a regular supply of samples from suitable patients. Engagement with clinicians involved attending multidisciplinary team meetings, giving seminars and updating the AWMGS website with information on the project. This approach facilitated good communication between the laboratory and lung oncologists which was vital to identify *EGFR*-positive patients who could donate samples for use in service validation.

One of the challenges with any service development is how to improve access and engagement at clinical centres further from the laboratory. The AWMGS did this by working with oncologists based in the central hospitals who also treated patients in clinics at peripheral hospitals.

Expansion of testing to peripheral centres also presented an opportunity to clarify issues of logistics such as:

- Transport of samples to the laboratory
- Ensuring contact information for the laboratory is easily available and accessible
- Where to get a test referral form and blood collection tube
- Instructions on how to collect a sample
- How to send the sample back to the laboratory
- Expected turn around time
- How reports are returned (to the requesting clinician via the NHS secure email network)

Once the service had been established, the challenge was to develop access beyond the initial group of engaged clinical referrers in South Wales and to expand the testing network to other Welsh hospitals and those in South West England. This required engaging with clinicians who were not as knowledgeable about ctDNA testing. The AWMGS took an extremely proactive approach and several members of the laboratory staff and clinical users visited other hospitals to communicate with oncologists in person about the service, or attended multidisciplinary team meetings (MDTs). As a result of these efforts the laboratory has significantly increased the reach of the service, and a number of respiratory physicians have expressed an interest in testing before meeting to discuss the patient at the MDT and are also interested in using testing as a diagnostic test at the initial investigation stage (refer to patient pathway in section 2.1). These efforts slowly increased the number of samples that the laboratory received and allowed it to further develop the service in a controlled manner.

The AWMGS took an extemely proactive approach to engaging clinicans about ctDNA testing. Several members of the laboratory staff and clinical users visited other hospitals to communicate with oncologists in person about the service, or attended multidisciplinary team meetings (MDTs). As a result of these efforts the laboratory has significantly increased the reach of the service

Clinician and patient responses to AWMGS efforts

Initial clinician responses to the establishment of the ctDNA testing service were positive – the test was perceived to be simple and quick and benefited patients through increasing access to a targeted therapy. Ease of testing and consistent turn around times enabled better communication with patients as to when they could expect their results and return to clinic to discuss them.

There were concerns, particularly over how to interpret negative results and when/how often to test. Access to drugs was also a concern as there is no Cancer Drugs Fund in Wales and for a time osimertinib could not be accessed. It was also unclear who pays for the test and drugs in some cases. Differences between England and Wales in terms of which drugs are funded and funding processes caused some confusion.

Patients responded positively to testing, not just because people preferred a blood test to a biopsy, but also recognised the potential to access better tolerated and targeted therapies. However, clinicians need tools to help them manage negative results, in terms of how they help the patient to deal with uncertainty and what the options are should a test be negative.

Lessons learned from AWMGS engagement activities

Patient access to testing could be affected by the level of interest their clinician has in current research - clinicians with a research background are more likely to know what research is ongoing and to understand the possible impact of the latest developments. This highlights the need to give clinicians a very clear message about what testing is for, how it will benefit patients and to develop tools to aid understanding of the relevance of sensitivity and specificity - what can affect test results, clinical utility, when and how often to test.

The success of engagement also relies on open and interactive professional relationships between the laboratories and clinicians. This open relationship facilitates the exchange of information including what the laboratory does and what it can offer, to enabling the clinicians sharing information with the laboratory to understand how the information they provide could improve the service.

5.4. Funding and commissioning

The NHS funding systems differ among the devolved nations of the UK.

The two main areas where stakeholders have reported funding challenges or confusion over funding sources are:

- **Funding for resources and members of staff to validate new tests/technologies** - this is an issue for NHS Trusts providing testing services
- **Funding structures for test payment** - in order to have a systematic roll out of this new NHS service it will be necessary for test providers to establish formal supply arrangements with clinical service providers including service contracts

Pharmaceutical companies provided support in terms of funding testing and other support such as data, expertise, equipment and consumables. The laboratories that have successfully implemented testing made use of a variety of internal and external funding sources to support test development. Clarity is needed as to the sources of potential support for test development and how these can be accessed, particularly in light of available but limited pharma funding.

Many stakeholders also report that funding structures for test payment are unclear. A stakeholder briefing jointly produced by the ABPI, BIVDA and CRUK[39] brings some clarity by outlining the NHS England commissioning and funding arrangements for six molecular genetic tests for cancer and also more established tests such as *HER2/ER* testing in breast cancer and *EGFR* testing in lung cancer. *EGFR* testing in lung cancer is currently reimbursed through the Health Resources Group tariff system and as such both solid and liquid tumour testing should be reimbursed. The processes by which Trusts can claim back the costs of tests is unclear to many and it is possible that this situation is contributing to some patients not having access to the tests that they need. Future engagement efforts will require:

- **Promotion of current funding** and reimbursement arrangements for *EGFR* testing to clinicians and hospital finance teams
- **Improved communication links** between NHS Regional Commissioning managers and hospitals

Procurement challenges

At a more local level, laboratories have encountered procurement challenges with the blood collection tubes needed to collect samples. Oncologists found that their departments did not have a specific budget for ordering tubes and struggled to procure them. The laboratories have therefore developed a system whereby they send the necessary tubes to the oncologists and include the cost of the tube (approximately £5) in the test price. This increases the cost of the test and the laboratory must carefully manage the supply according to the number of patients and clinics, while also considering the shelf-life of the tubes. While this arrangement is more burdensome for the laboratories (they send the tube to the oncologist before sample collection) this approach does ensure that the correct equipment is available.

5.5. Looking to the future – extending the use of testing in lung cancer

Having made the investment and developed a clinical *EGFR* testing service, how can the NHS make the most of the positive impact on patients and extend the clinically appropriate use of the technology within lung cancer care? Are there areas where testing could be expanded?

Laboratories currently only offer ctDNA *EGFR* testing after diagnosis of lung cancer in two situations:

1. When a patient has a diagnosis of lung cancer but there was not enough material in the solid tumour biopsy for a genetic test, or the genetic test failed
2. On progression on TKIs, to test for the p.T790M mutation

Potential for using ctDNA testing in lung cancer

Monitoring patients after surgery, to determine risk of relapse

This technique is currently under investigation in clinical research studies – for example, one of the objectives of the TracerX study is to use ctDNA testing and other methods to explore the relationship between tumour heterogeneity and clinical outcomes after surgery in NSCLC patients.

Use as a 'gateway' technology

As a gateway technology, ctDNA testing could be used to determine if a patient is a good candidate for more expensive invesitigation e.g. CT scan. For example, it has been demonstrated that ctDNA testing can identify patients with diffuse large B-cell lymphoma who are at risk of recurrence before clinical evidence of disease is detected using CT scans, suggesting that ctDNA testing could be used as an adjunct to scanning technology or to identify patients who should be monitored more closely[40].

Monitoring patients on TKIs to detect emergence of resistance

Currently, patients are tested for the p.T790M mutation once tumour progression has been detected on a scan; ctDNA testing could be used to monitor patients and detect the emergence of p.T790M and switch therapy earlier, before the tumour has changed size. However clinical evidence is lacking as to whether this is beneficial to patients in terms of progression-free and overall survival.

Screening and early detection

One of the more controversial uses of ctDNA is its potential use in screening and early detection. Most of the uses outlined above occur in patients in whom a definitive diagnosis has happened by alternative methods. In order to use ctDNA effectively for early detection and screening, the user needs to know which mutations to look for (to distinguish ctDNA from healthy cfDNA) and also which tissue they came from.

Determining tissue of origin using ctDNA is possible but technically challenging and a long way from being ready for clinical use[41]. Should a patient without a cancer diagnosis have a ctDNA screening test that uses a panel test to search for the full spectrum of known cancer mutations, and a positive hit occurs, it is unclear what should happen next. Firstly, it will not be clear what is producing this mutation positive DNA without further extensive testing to determine if a tumour is present. Secondly, what are the clinical implications of finding a mutation in cfDNA in the blood? Is the source harmful or benign? Are these mutations clinically relevant and do clinicians have a clear treatment pathway for managing patients with positive test results? There is therefore a danger of over-investigation and over-treatment with the associated worry for patients should a test be positive. Much more work is needed to determine how ctDNA could be used effectively as a screening tool in a variety of cancers, including lung.

Funding multiple ctDNA tests

More extensive use of testing, for example as a monitoring tool in various scenarios, presents a challenge since this use does not fit into the conventional 'one test, one payment' model. How such testing can be funded will need to be addressed.

Beyond lung cancer

Evidence is developing to support the clinical use of ctDNA testing for *KRAS* mutations in colorectal cancer (anti-EGFR therapies can be effective in *KRAS* wild-type patients) and *BRAF* in melanoma (therapies targeting p.V600E mutation).

How can the NHS maximise the utility of emerging ctDNA services?

- Clinical guidelines are needed on the use of ctDNA testing, and clinician and laboratory expertise should be actively collected to inform their development
- Ongoing external quality assurance efforts will help to answer the question of appropriate levels of test quality and performance
- Engagement with and within the health system about testing will be needed to increase awareness about testing and how it can be used to benefit patients
- Service establishment and validation can be supported by promotion of available funding, promotion of test funding structures, linking of test development into accelerated access of technologies and support of collaborative test development

6. What needs to be done to support service implementation

We have shown that there is growing evidence for the use of ctDNA testing in the treatment of lung cancer. Such testing has increased patient access to tumour genetic testing and therefore to targeted therapies which improve progression-free survival compared to standard chemotherapy. NHS services for ctDNA testing are currently available from a select number of laboratories in the UK, offering ctDNA tests for *EGFR* mutation status in eligible patients with NSCLC.

The main challenges that lie ahead are to:

1. Improve the quality of tests through technical development and further research into the biology of ctDNA
2. Ensure that more eligible patients receive testing

Much progress has already been made and there is now a valuable opportunity for NHS England and other stakeholders to accelerate access to this transformative technology. However, if these opportunities are not seized now, it is likely that patients will be subjected to a postcode lottery in terms of access to testing and therefore to innovative therapy, which will have a knock-on negative effect on patient outcomes in lung cancer.

We outline below measures that can be taken to support further development and awareness of ctDNA testing such that all patients can benefit from the efforts of test pioneers. While there are many areas with potential for improvement, priorities should be to:

1. Improve and strengthen current services
2. Carry out engagement with and within the health system about testing

Improve and strengthen current services

The laboratories currently offering testing have invested considerable resources, time and expertise into developing their services.

Currently much of the expertise in ctDNA testing is concentrated in academic centres and in regional laboratories. Concentrating expertise and continuing technology development in these laboratories will:

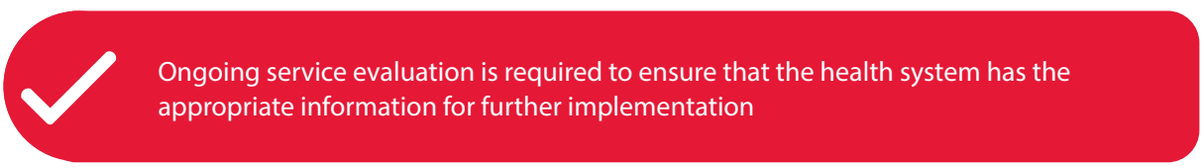
1. Make the most of the opportunities presented to learn from research expertise and technology development, which in turn will contribute to the development of guidelines
2. Enable these laboratories to more easily take part in clinical research studies and closely collaborate with researchers – there is an opportunity to take advantage of initiatives already in place such as the CRUK Experimental Cancer Medicine Centres
3. Provide an opportunity to resolve outstanding issues before the wider roll-out of ctDNA testing beyond lung cancer, which is expected in the next few years

Ongoing NHS service evaluation efforts will also have a role to play by ensuring that the health system has the appropriate information needed for further implementation. Continuing collaboration will be needed between laboratories, clinicians and other stakeholders to resolve outstanding questions that arise as a result of these evaluations.

Advantages of this approach

In broader terms, organisation into services and 'hub and spoke' models of delivery fits with the planned reconfiguration of genomic laboratory services in England. Given that many oncology services operate in this way, with central hospitals providing specialist testing for peripheral hubs, there is an opportunity to develop processes to ensure consistency of delivery. Currently ctDNA testing volumes in NSCLC are relatively low, which supports focusing provision in a defined number of centres.

Any clinical services considering using ctDNA testing services should consider using services already provided by a specialist laboratory centre rather than establishing their own.

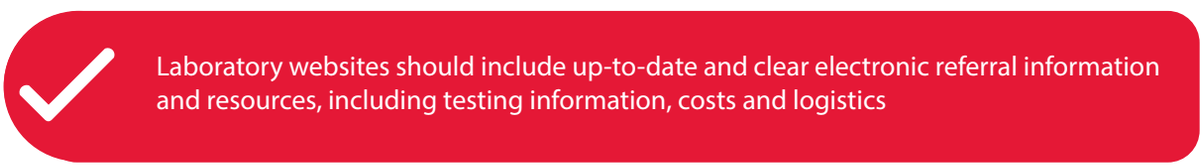


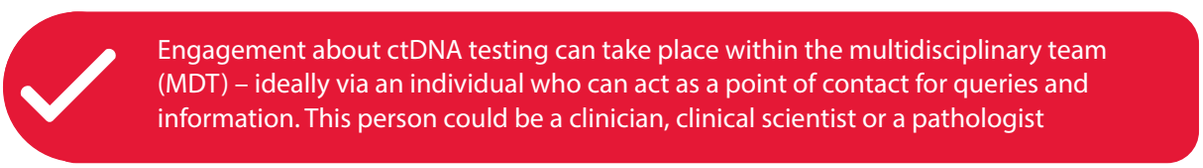
Engagement with and within the health system

The establishment of current services has demonstrated the role of clinician leadership in the development of services. There is an opportunity to capitalise on the expertise that already exists within the clinical community and the laboratories to build a network of clinical champions who can support the development of ctDNA testing in NSCLC.

How might this happen and who would be involved?

- **Engagement of clinical champions** who are knowledgeable about and are already using ctDNA testing
- **Making use of local networks** where clinical champions can promote testing as an option for patients e.g. through MDTs, regional clinical meetings
- **Engaging the professional societies and other organisations to consider and develop clinical guidelines** for ctDNA testing in order to engage those who might have less awareness of testing and what it could be used for e.g. British Thoracic Oncology Group, British Thoracic Society, Royal College of Pathologists, Cancer Research UK (including the ECMC network), NICE (clinical guidelines for lung cancer). As outlined in section 3.1, the Scottish Medicines Consortium is the only UK organisation that has guidelines on the use of ctDNA testing in NSCLC





The experience of the All Wales Medical Genetics Service demonstrates one way that engagement can work in practice. While initial engagement is demanding in terms of time and effort, the strategy has succeeded in terms of increasing knowledge amongst clinicians spread over a wider geographical area, which in turn creates a positive feedback loop for the laboratory by increasing demand.

Development of community-led guidelines

As laboratories and clinicians expand their expertise in using testing they will be gaining knowledge on test performance and use in a clinical setting. This knowledge should be actively collected and inform guideline development and updates.

What questions could be addressed by guidelines?

- **Test sensitivity -** clear explanation and guidance on the limitations of testing compared to solid biopsy (which will change as technological improvements are made)
- **Negative results -** how to report negative results; what negative results mean; clear procedures to follow in the case of negative results e.g. retest, proceed to solid biopsy
- **When to test -** when in the patient journey is best to perform a test; in the case of negative results, when to retest; how often should retesting occur
- **Patient information -** information for patients about testing, what it does and why it is offered, the meaning of positive and negative results and the impact that this will have on their treatment

Clinical guidelines on the use of ctDNA testing in NSCLC should be developed by one or more of the professional societies and organisations, such as: British Thoracic Oncology Group, British Thoracic Society, Royal College of Pathologists, Cancer Research UK (including the ECMC network), NICE (clinical guidelines for lung cancer). Clinician and laboratory expertise in ctDNA testing should be actively collected to inform these guidelines

What can the health system do to support services?

Support for service establishment and validation

Laboratories made use of a range of funding sources to develop current services in some cases to fund equipment and an additional member of staff to develop and validate techniques, in the absence of additional funding being available there could be:

- **Promotion of the sources of funding** already available to support technology development within laboratories
- **For validated testing services, promotion of the test funding structures already in place** – a stakeholder briefing by ABPI, BIVDA and CRUK outlines commissioning and funding arrangements for six molecular genetic tests for cancer and also highlights other tests that are included in the tariff[39]
- **Linking of test development into accelerated access of technologies** and the findings of the accelerated access review
- **Support of collaborative test development** by laboratories to avoid duplication of effort

Service establishment and validation should be supported, by and within the health system, by promotion of available funding, promotion of test funding structures, linking of test development into accelerated access of technologies and support of collaborative test development

Vision for laboratories – to what extent should laboratories drive the implementation of new technologies?

Development of new tests is inherently risky. As experts in this field, laboratories make informed decisions about the risks versus the benefits of implementing new technologies giving them a level level of control over which new technologies they think will have the greatest clinical impact. This presents an opportunity for laboratories to take the lead in this area, taking on some risk but also reaping the rewards for doing so. As the use of ctDNA testing expands beyond *EGFR* and lung cancer, the network of current ctDNA providers have the opportunity to build on the foundations that are already in place and to strengthen and consolidate the services they are offering.

How does ctDNA testing fit into the bigger picture?

One of the goals of the NHS England Cancer Strategy is to find cancer early and cure it early. Future uses of ctDNA might have more of an impact in helping to meet these goals, for example through use in screening or monitoring patients after treatment. ctDNA could also be used as a 'gateway' test to more expensive technology e.g. CT scans. However much research still needs to be done to investigate the use of ctDNA in these areas.

Link science, test development and drug availability

For TKIs, the science in terms of drug and test development was ahead of clinical practice. Targeted drugs and testing had been developed, however there was a delay to drugs being funded. In the case of osimertinib, ctDNA testing was available in Wales, but the drug was not, since Wales is not covered by the Cancer Drugs Fund, however patients could access the drug through Individual Patient Funding Requests.

NHS England should consider how patients can have improved access to funded targeted therapies and take steps through policy development to ensure that the health system is better prepared to implement targeted therapies when commissioned by the NHS

7. Conclusions

Circulating tumour DNA testing in NSCLC increases access to targeted therapies and is meeting an unmet clinical need. In this report we have presented evidence for and made recommendations on three key questions for the NHS:

Should the NHS offer ctDNA testing of EGFR in lung cancer to all eligible patients?

Yes, the evidence of clinical utility demonstrates that ctDNA testing makes a difference to patients, increasing accessibility to targeted therapies. While some improvements are needed to techniques, these are not a barrier to adoption, since the alternative is that patients will not have testing and the opportunity to access targeted therapies.

How should the NHS use ctDNA testing to maximise access to TKIs?

The health system should support existing services – ctDNA testing at diagnosis and for p.T790M – and establish current services as centres of excellence for ctDNA testing in lung cancer. Clinical guidelines from NICE, or developed by the community, are crucial for raising awareness and providing reassurance to clinicians that these tests have clinical utility and benefit patients.

What lessons can be learned from existing ctDNA services to support the wider use of ctDNA tests in future?

Active engagement efforts are effective, as demonstrated by the AWMGS, but require the laboratories to invest time and resource. Further efforts such as these will be required to improve engagement within the health system. Learning from early pioneers and implementing comprehensive and equitable ctDNA testing for NSCLC now will be an investment for the future, when more uses of this technology are likely to become available.

It seems likely that ctDNA testing is here to stay and that its use in cancer care will increase as technological improvements are made and more clinical evidence is gathered to support its use in different stages of the patient pathway. If this potential is to become a reality, the health system should take the opportunity to build the foundations now to support future test implementation.

8. Recommendations

- Healthcare commissioners should formally consider the provision of ctDNA services in lung cancer and improve and strengthen current service provision
- Ongoing service evaluation is required to ensure that the health system has the appropriate information for further implementation
- Laboratory websites should include up-to-date and clear electronic referral information and resources, including testing information, costs and logistics
- Engagement about ctDNA testing can take place within the multidisciplinary team (MDT) – ideally via an individual who can act as a point of contact for queries and information. This person could be a clinician, clinical scientist or a pathologist
- Clinical guidelines on the use of ctDNA testing in NSCLC should be developed
- Service establishment and validation should be supported, by and within the health system, by promotion of available funding, promotion of test funding structures, linking of test development into accelerated access of technologies and support of collaborative test development
- NHS England should consider how patients can have improved access to funded targeted therapies and take steps through policy development to ensure that the health system is better prepared to implement targeted therapies when commissioned

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10. Appendix

Speakers and attendees of the workshop held on 7th March 2017 at the Royal College of General Practitioners, London

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Prof Fiona Blackhall	Thoracic Oncology Chair / Consultant in Medical Oncology	University of Manchester / The Christie NHS Foundation Trust
Dr Rachel Butler	Head of Genetics Laboratory	Cardiff and Vale University Health Board
Dr Qamar Ghafoor	Clinical Oncologist	University Hospital Birmingham
Dr Daniel Nelmes	Oncology SpR	Velindre Cancer Centre, Cardiff
Dr Philippe Tanière	Consultant Histopathologist	Queen Elizabeth Hospital Birmingham
Dr Andrew Wallace	Consultant Clinical Scientist	St Mary's Hospital / Manchester Centre for Genomic Medicine

Delegate	Title	Organisation
Dr Hilary Burton	Director	PHG Foundation
Dr Sandi Deans	Director	UK NEQAS for Molecular Genetics
Dr Tim Forshew	Head of Technology Development	Inivata Ltd.
Dr Louise Gaynor	Policy Intern	PHG Foundation
Dr Susan Harden	Consultant Clinical Oncologist	Cambridge University Hospitals
Dr Rob Hastings	Principal	Precision Medicine Catapult
Dr Crispin Hiley	Academic Clinical Lecturer	King's College London
Dr Said Isse	Consultant Respiratory Physician	Mid Essex Hospitals

Delegate	Title	Organisation
Dr Amy Jones	Principal Scientist	Haemato-oncology diagnostic service, Cambridge University Hospitals
Dr Maria Kapi	Senior Medical Advisor	Roche Diagnostics
Dr Mark Kroese	Deputy Director	PHG Foundation
Dr Angus Lauder	Associate Director, Business Management	Cancer Research Technology Ltd.
Dr Leila Luheshi	Head of Science	PHG Foundation
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Prof Clive Morris	Chief Medical Officer	Inivata Ltd.
Mark Richards	Director, Global Product and Portfolio Strategy, Oncology	AstraZeneca
Dr Rowena Sharpe	Head of Precision Medicine	Cancer Research UK
Dr Matthew Smith	Clinical Scientist	Queen Elizabeth Hospital Birmingham
Dr Lisa Thompson	Head of Molecular Diagnostics	The Royal Marsden
Laura Timm	Diagnostic Manager (Lung)	AstraZeneca
Dr Mikel Valganon	Senior Research Associate	University of Cambridge

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Liquid Biopsy in Non-Small Cell Lung Cancer

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Liquid biopsy analyses are already incorporated in the routine clinical practice in many hospitals and oncology departments worldwide, improving the selection of treatments and monitoring of lung cancer patients. Although they have not yet reached its full potential, liquid biopsy-based tests will soon be as widespread as "standard" biopsies and imaging techniques, offering invaluable diagnostic, prognostic, and predictive information. This review summarizes the techniques available for the isolation and analysis of circulating free DNA and RNA, exosomes, tumor-educated platelets, and circulating tumor cells from the blood of cancer patients, presents the methodological challenges associated with each of these materials, and discusses the clinical applications of liquid biopsy testing in lung cancer.

Keywords: ctDNA, ctRNA, CTCs, exosomes, tumor-educated platelets, mutations, gene fusions, lung cancer

INTRODUCTION

The so-called "liquid biopsy" is quickly moving from research into clinical practice in lung cancer, as well as in other human malignancies. Although its full potential has not yet been reached, the "liquid biopsy" is no longer a promise but a reality that is allowing a better treatment selection and monitoring of lung cancer patients in hospitals and oncology departments worldwide. We can already foresee a day when "liquid biopsy"-based tests will be as widespread and useful as "standard" biopsies and imaging techniques, offering invaluable diagnostic, prognostic, predictive, and monitoring information. In this mini review, we will summarize the state of the art in this exciting area, placing a particular emphasis on the clinical utility of the "liquid biopsy" and the variety of applications, methodologies, and results that can be derived from it.

"Liquid biopsies" are usually defined as tests done in blood samples or other body fluids. In the case of cancer patients, the objective of those tests is to detect materials originated in the tumor. Although the term "liquid biopsy" is universally used, many pathologists argue that it is incorrect. The so-called "liquid biopsies," they claim, are not true biopsies. A "true" biopsy is usually performed by a surgeon or a pneumologist and involves the extraction of sample cells or tissues that are subsequently examined by a pathologist under a microscope, commonly after some kind of fixation and staining. Paraffin embedding is also widespread. In contrast, "liquid biopsies" are not obtained by surgeons; involve the extraction of blood or other fluids and not of solid tissues, pathologists only

occasionally intervene and fixation, embedding, or staining are equally infrequent. In addition to the "biopsy" half, the "liquid" half in the term "liquid biopsy" can also be misleading. The materials originated in the tumor that are to be detected in such "biopsies" are never liquid. Some of them are cells or fragments of cells, such as circulating tumor cells (CTCs), exosomes, or tumor-educated platelets (TEPs); others are nucleic acids dissolved in the blood, such as circulating tumor DNA or RNA (ctDNA, ctRNA). Each of these materials offers unique opportunities to test different biomarkers and analyze particular characteristics of the tumors (**Table 1**).

The differences between a "real" and a "liquid" biopsy—or "liquid sample," as the pathologists would probably prefer to call them—explain the advantages of the latter. "Liquid" biopsies will never replace real biopsies, which are irreplaceable sources of information that cannot be obtained by any other means, such as tumor type and histology. However, they offer all sorts of additional data that cannot be obtained in any other way. In patients who cannot be biopsied, or where biopsies do not have enough tissue, "liquid biopsy" is the only alternative to perform genetic testing for targeted therapy. Also, in patients with advanced disease, it is not feasible to obtain biopsies of every metastasic site. But blood reaches both the primary tumor and the metastases, and materials coming from all can be found in a "liquid biopsy." Finally, unlike "real" biopsies, blood can be repeatedly obtained without the risk of comorbidities and used to monitor the course of the disease, including early detection of response and relapse or emergence of resistance to a particular therapy.

CIRCULATING TUMOR DNA

Circulating free DNA (cfDNA) can be found dissolved in plasma and serum, at variable amounts. In the case of cancer patients, a fraction of the cfDNA is tumor derived, and ctDNA represents from less than 0.1% to more than 10% of the total cfDNA. This percentage has been shown to depend on stage, tumor burden, vascularization of the tumor, biological features like apoptotic rate and metastatic potential of the cancer cells, and factors affecting the blood volume of the patient (1, 2). In addition, variations on the relative abundance of ctDNA correlate with response to therapy (3–5). ctDNA is released by passive mechanisms, such as lysis of apoptotic and necrotic cells or digestion of tumor cells by macrophages, and also by active mechanisms. In this respect, cfDNA shows and enrichment in 150–180 bp fragments typical of the nucleosomal pattern of DNA fragmentation during apoptosis (6–9). The ctDNA carries the same somatic alterations as the tumor itself and can be used to detect clinically relevant mutations such as those in the epidermal growth factor (*EGFR*) or *KRAS* genes. This is particularly useful when no biopsy is available for genetic analyses and, in this setting, the European Medicine Agency recommends *EGFR* testing in liquid biopsies to select patients for tyrosine kinase inhibitor (TKI) therapy (10). However, many standard techniques for mutation detection are not useful for ctDNA analyses due to an insufficient sensitivity. Since ctDNA often represents a small percentage of the total cfDNA, somatic mutations coming from the tumor can be present at allele fractions as low as 0.01%. Highly sensitive methodologies, or variations of preexisting methodologies, have been developed in order to detect low abundance mutations in cfDNA (6, 11).

TABLE 1 | Biological materials that can be isolated from liquid biopsies and their applications in lung cancer.

Material	Applications
Circulating tumor DNA (ctDNA)	Somatic mutations[a] DNA methylation changes Copy number alterations
ctRNA	Gene fusion Splicing variants
Tumor-educated platelets	Gene fusions Splicing variants Cancer diagnosis RNA profiling
Exosomes	Gene fusions Splicing variants miRNA analyses RNA and protein-based molecular profiling
Circulating-tumor cells (CTCs)	Monitoring (total CTC counts)[b] Culture of CTCs DNA, RNA, and protein-based molecular profiling Somatic mutations Gene fusions

Applications used in routine clinical practice in ([a]) NSCLC or ([b]) metastatic breast, prostate, and colon cancer patients. Unmarked, research use.

Modified real-time PCR techniques have been widely used to identify genetic alterations in the cfDNA of cancer patients. They include amplification-refractory mutation system [ARMS (12),], Scorpion-ARMS (13), and peptide nucleic acid (PNA) or locked nucleic acid (LNA) mutant-enriched PCR (14–17). The diagnostic sensitivity of these techniques, when compared to tumor tissue, ranges from 43 to more than 90%, while the specificity is usually close to 100%; and the two commercially available methods to determine *EGFR* mutations in the cfDNA of cancer patients (Therascreen Plasma from Qiagen and COBAS Blood from Roche Diagnostics) are based on them. In our group, we have developed a quantitative PCR technique in the presence of PNA to detect *EGFR*, *KRAS*, and BRAF mutations in the cfDNA of advanced lung, colon, and cancer patients that achieves 75–80% sensitivity with 100% specificity (18, 19). Digital PCR, droplet digital PCR, and beads, emulsion, amplification, and magnetics (BEAMing) system constitute further refinements of the PCR-based techniques and have also been used to determine mutations in cfDNA (14, 20–26) (**Table 2**).

Most modified PCR techniques are easy, comparatively unexpensive, and have a quick turnaround time (19), but have the disadvantage that can only detect mutations in a limited number of loci, usually within a single gene. Next-generation sequencing methodologies can overcome these limitations but, while tissue-based NGS genotyping is already well established, the application of NGS technologies to liquid biopsies is challenging and an ultra-deep sequencing approach is commonly used in order to improve sensitivity. In this approach, the gene panels are limited so that each read is sequenced thousands of times (39, 50, 51). However, this requirement of a high sensitivity may easily lead to false-positive results and requires a careful

TABLE 2 | Summary of reports on detection of genetic alterations in liquid biopsy materials from advanced NSCLC patients.

Technique	*n*	Type of sample	Alteration detected	Sensitivity (%)	Reference
ARMS	86	Circulating free DNA (cfDNA) (plasma)	Epidermal growth factor (EGFR)-sensitizing mutations	68	(27)
SARMS	42	cfDNA (serum)	EGFR-sensitizing mutations	75	(13)
SARMS	11	cfDNA (serum)	EGFR-sensitizing mutations	50	(13)
SARMS	21	cfDNA (plasma)	EGFR-sensitizing mutations	39	(28)
SARMS-based DxS EGFR mutation test kit	86	cfDNA (serum)	EGFR-sensitizing mutations	43	(15)
SARMS-based EGFR mutation detection kit	652	cfDNA (plasma)	EGFR-sensitizing mutations	66	(12)
Mass spectrometry-based genotyping	31	cfDNA (plasma)	EGFR-sensitizing mutations	39	(29)
Mutant-enriched PCR			EGFR-sensitizing mutations	33	
Mutant-enriched PCR	18	cfDNA (plasma)	EGFR-sensitizing mutations	100	(30)
Mutant-enriched PCR	111	cfDNA (plasma)	EGFR-sensitizing mutations	56	(31)
EGFR array, PNA-PCR	37	cfDNA (plasma)	EGFR-sensitizing mutations	100	(32)
Digital PCR	35	cfDNA (plasma)	EGFR-sensitizing mutations	92	(22)
Droplet digital PCR	46	cfDNA (plasma)	EGFR-sensitizing mutations	67	(33)
Droplet digital PCR	50	cfDNA (plasma)	EGFR mutations	76	(34)
Droplet digital PCR	25	cfDNA (plasma)	EGFR mutations	81	(35)
Cobas® EGFR blood test	199	cfDNA (plasma)	EGFR-sensitizing mutations	61	(20)
Cobas® EGFR blood test	38	cfDNA (plasma)	p.T790M (EGFR)	73	(36)
Cobas® EGFR blood test	238	cfDNA (plasma)	EGFR mutations	76	(14)
DHPLC	230	cfDNA (plasma)	EGFR-sensitizing mutations	82	(37)
DHPLC	822	cfDNA (plasma)	EGFR-sensitizing mutations	77	(36)
BEAMing	44	cfDNA (plasma)	EGFR-sensitizing mutations	73	(24)
BEAMing[b]	915	cfDNA (plasma)	EGFR, KRAS, BRAF, PIK3CA mutations	83–99[c]	(23)
BEAMing	153	cfDNA (plasma)	EGFR-sensitizing mutations p.T790M	82 73	(26)
Cobas® EGFR blood test			EGFR-sensitizing mutations p.T790M	73 64	
PNA-Q-PCR	97	cfDNA (serum/plasma)	EGFR sensitizing mutations	78	(18)
PNA/LNA-Q-PCR	35	cfDNA (serum)	EGFR, KRAS mutations	73	(17)
NGS (CAPP-Seq)	142	cfDNA (plasma)	EGFR mutations	81	(38)
NGS (Ion Torrent)[a]	107	cfDNA (plasma)	EGFR, HER2, KRAS, BRAF, PIK3CA mutations	58	(39)
NGS (deep sequencing)	288	cfDNA (plasma)	EGFR mutations	73	(40)
Melting curve PCR	8	Circulating tumor cells (CTCs)	EGFR mutations	100	(41)
NGS	37	CTCs	EGFR mutations	84	(42)
Mutant-enriched PCR	21	CTCs	p.T790M (EGFR)	57[c]	(43)
	25	cfDNA (plasma)		60[c]	
ISET + fluorescence *in situ* hybridization (FISH)	5	CTCs	ALK fusions	100	(44)
ISET + filter-adapted FISH	32	CTCs	ALK fusions	100	(45)
ISET + filter-adapted FISH	4	CTCs	ROS1 fusions	100	(46)
Antibody-independent CTC isolation + FISH	31	CTCs	ALK fusions	≥90[c]	(47)
NanoVelcro System + FISH	41	CTCs	ALK fusions	100	(48)
Retrotranscription PCR	77	cfRNA (plasma)	ALK fusions	22	(49)
		Platelets	ALK fusions	65	

[a]*Samples in the study include stages I–IIIA.*

[b]*Samples in the study include tumors other than NSCLC.*

[c]*Concordance value.*

validation of the whole testing process. Examples of NGS protocols specifically developed for ctDNA analysis include TAm-Seq (tagged-amplicon deep sequencing), which combines site-specific primers with universal tails (52, 53); Safe-SeqS (Safe-Sequencing System) (54), and CAPP-seq (capture based sequencing), which relies on hybridization-based capture of target regions followed by amplification (38, 55) (**Table 2**).

The detection of mutations in cfDNA by modified PCR or NGS techniques is not only useful in lung cancer patients at presentation. It has also been successfully employed for patient monitoring, including early evaluation of response and relapse, which are associated with changes in the *EGFR* or *KRAS* mutational burden in cfDNA; and for early detection of acquired resistance to EGFR TKIs, associated in many patients with the emergence of the p.T790M mutation in blood (26, 56). In this respect, p.T790M testing in cfDNA has been recently recommended in patients eligible for osimertinib treatment, in order to avoid unnecessary rebiopsies (33, 36, 56).

CIRCULATING TUMOR RNA

Similar to ctDNA, RNA derived from tumor cells (ctRNA) is present in the plasma of cancer patients and can be used for detection of the clinically relevant *ALK*, *ROS1*, and *RET* fusion genes and METΔ14 splicing variant. However, genetic analyses in cfRNA present specific challenges and have not been widely used. Unlike cfDNA, cfRNA degrades very quickly and needs to be purified rapidly after blood extraction. The alternative is adding a preservative such as Trizol and freezing the sample at −80°C, but this procedure is not easily accessible to many clinical sites. Despite these limitations, our group has a 5-year experience in detection of *EML4-ALK* fusion transcripts in plasma cfRNA by retrotranscription PCR (RT-PCR) (49) and, using improved processing and purification methods, we have demonstrated that the sensitivity of the technique can be significantly improved.

TUMOR-EDUCATED PLATELETS

Platelets have been recently demonstrated to sequester tumor RNA by a microvesicle dependent mechanism, and the so-called TEPs (57, 58) can be used as a source of tumor RNA for genetic analysis. Platelets can be isolated from blood by simple centrifugation steps, and its RNA content easily purified and used for the detection of gene fusions and splicing variants. Using a RT-PCR approach, our group has detected *EML4-ALK* fusion transcripts in TEP RNA from advanced lung cancer patients with 65% sensitivity and 100% specificity (49). In addition, we have demonstrated that the disappearance of fusion transcripts in platelets correlates with response to crizotinib treatment. Regarding splicing variants, the clinical relevance of METΔ14 in lung cancer was only described in 2015 (59–61), and there are no reports in the literature about detection of METΔ14 transcripts in liquid biopsy. However, we have recently detected the alteration in the TEP RNA of a NSCLC patient positive in tumor tissue, who attained a partial response to crizotinib (unpublished data).

Platelet RNA can also be analyzed by multiplexing techniques, and a recent report has demonstrated the diagnostic potential of this approach. Using mRNA sequencing and surrogate TEP RNA profiles of 283 samples, 228 cancer patients of six different origins were discriminated from 55 healthy individuals with 96% accuracy. Tumors with specific genetic alterations, such as *KRAS* or *EGFR* mutations, were also distinguished and the location of the primary tumor identified with 71% accuracy (58).

EXOSOMES

Exosomes are small vesicles present in blood and other body fluids (62–64). With a 30–100 nm diameter, they are constitutively released through exocytosis by many cells, including tumor cells, in physiological and pathological conditions. Exosomes contain lipids, proteins, mRNA, several types of non-coding RNAs, and double-stranded DNA; and their composition partly reflects that of the parental cells (65). In addition, being generated by the cell secretion pathway, all exosomes carry some common proteins independent of their origin, such as ALIX, CD63, or TSG-101 (66). Exosomes are generally isolated by sucrose gradient ultracentrifugation or immune-bead isolation techniques (such as magnetic activated cell sorting), and there are commercial kits available. Once isolated, exosomes are characterized by transmission electron microscopy, Western blot, FACS, or other methodologies (67).

Although being more difficult to purify than other materials, the lipid bilayer of exosomes makes their cargo particularly stable, theoretically allowing the identification of the tumor of origin, genetic alterations or resistances to treatments. In this respect, EML4-*ALK* fusion transcripts have been recently identified in the exosomal RNA of NSCLC patients (68). In addition, some studies indicate that micro RNA (miRNA) analysis of exosomes might be useful for the diagnosis of lung adenocarcinoma (69–71) and that particular miRNAs can offer prognostic information in advanced NSCLC. For example, downregulation of miRNA-373 and miRNA-512 has been associated with a poor prognosis (72), miR-208a and miR-1246 with resistance to radiotherapy (73, 74), and miR-221-3p and 222-3p with good response to osimertinib in *EGFR* mutated patients (75).

CIRCULATING TUMOR CELLS

Together with ctDNA, CTCs are the most widely investigated material in liquid biopsies of cancer patients. First observed in 1869 (76), they are cancer cells detached from the solid tumor mass that circulate in the blood and lymphatic system (77) as single cells or as aggregates, the so-called circulating tumor microemboli (78–80). In advanced NSCLC patients, CTCs are relatively rare, 1–10 per mL against a background of 10^6–10^7 peripheral blood mononuclear cells. This low abundance poses formidable challenges for the development of robust and sensitive enrichment protocols (81).

Some CTC capture methods are label dependent, based on specific epithelial cell surface markers, such as epithelial cell adhesion molecule (EpCAM) for positive selection or CD45 for negative depletion. One of such techniques, the CellSearch® system (Veridex), has been approved by the FDA for monitoring some type of tumors (82–84), but not lung cancer. In advanced NSCLC, CellSearch® has shown a limited detection efficiency, with CTCs detectable in only 20–40% of patients (85–87). Label-dependent methods do not select CTCs that have undergone epithelial to mesenchymal transition (88) or those with stem cell characteristics that have not started epithelial differentiation. Label-independent techniques, which are based on physical characteristics such as size, can overcome this limitation. Isolation by Size of Epithelial Tumor cells (ISET®, Rarecells), based on filtration and cytological characterization, has shown an increased sensitivity in NSCLC (89–92) with an 80% detection rate of CTCs in blood from 40 stage IIIA–IV patients compared with 23% using CellSearch® (85). Another technology based on size, ScreenCell®, allows not only the detection but also the isolation of CTCs, which can be subjected to further morphological studies and used for genetic testing. Isolated CTCs can be cultured or injected into mice to generate xenografts (93–96) and CTC-derived tumor cells can thus be obtained in enough numbers for molecular and pharmacological profiling.

CTC counts have been extensively researched as a prognostic factor in NSCLC (97). In early-stage patients, the decrease or disappearance of CTCs after surgery has been reported to correlate with better clinical outcomes (98, 99), while its persistence was associated with shorter progression-free survival (PFS) (100). Regarding advanced NSCLC, some studies have reported that a higher CTC count at presentation correlates with advanced stage and shorter PFS and overall survival (85, 101). Also, the decrease or disappearance of CTCs after chemotherapy or targeted therapy has been consistently associated with better outcomes (102–104).

Finally, CTCs have also been investigated as a tool to identify clinically relevant genetic alterations in NSCLC (**Table 2**). Using NGS and modified PCR techniques, *EGFR*-sensitizing mutations and the p.T790M resistance mutation have been detected in the CTCs of *EGFR*-positive patients at presentation and after progression to TKI treatments, respectively (28, 41, 42, 105). The sensitivities reported range from 47 to 100%. However, unlike cfDNA, CTCs are not used for *EGFR* testing in the routine clinical practice. EML*4*-*ALK* fusions have been identified by fluorescence *in situ* hybridization (FISH) and immunochemistry in CTCs isolated using ISET (44) or other enrichment methodologies (47, 48). In some cases, filter-adapted FISH was employed, a methodology that has also been demonstrated to successfully identify ROS1 rearrangements in CTCs isolated by ISET (46).

AUTHOR CONTRIBUTIONS

All the authors contributed to the writing and critical revision of the review.

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Review Article

Critical issues in the clinical application of liquid biopsy in non-small cell lung cancer

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Abstract: Current therapeutic options for non-small cell lung cancer (NSCLC) patients are chemotherapy and targeted therapy directed mainly against epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements. Targeted therapy relies on the availability of tumor biopsies for molecular profiling at diagnosis and to longitudinally monitor treatment response and resistance development. Unfortunately, tumor biopsy might be invasive, recover poor material of suboptimal quality, and cause sample bias due to tumor heterogeneity. Many studies have illustrated the potential of liquid biopsy as minimal invasive approach to respond to the urgent need for real time monitoring, stratification, and personalized optimized treatment in NSCLC patients. In principle, the liquid biopsy could provide the genetic landscape of primary and metastatic cancerous lesions, detecting "druggable" genomic alterations or associated with treatment resistance. Moreover, it would guarantee the prognostic/predictive biomarkers evaluation in patients for whom biopsies are inaccessible or difficult to repeat. At this regard, the prognostic value of circulating tumor cells (CTCs) in NSCLC patients has been largely investigated, but still their clinical utility as tumor biomarker is hampered by the lack of a consensus on the criteria necessary and sufficient to define them and on the standard operating procedures (SOPs) for their assessment. This review will summarize current developments on liquid biopsy in NSCLC, addressing the technology issues that contribute to the poor ability to track CTCs in the blood of NSCLC patients, thus limiting their extensive use in the clinical practice, and analyzing the solutions adopted to overcome such limits, on the road towards the clinical validation.

Keywords: Non-small cell lung cancer (NSCLC); liquid biopsy; circulating tumor cell (CTC); ctDNA, tumor marker

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Introduction

On the road from the laboratory bench to the patient's bedside, an assay measuring a tumor marker must be evaluated in order to demonstrate its analytic and clinical validity (respectively: the assay accuracy and reproducibility, and its ability to identify a biologic difference that predicts patient outcome), and at the end its clinical utility (the results of the assay—positive or negative—lead to a clinical decision that evidently improves patient outcome) (1). Thus, a tumor marker must encompass several levels of evidence in order to demonstrate its clinical utility, with the final aim to improve patient outcome, in terms of overall survival (OS), disease-free survival and quality of life, and obtain a more cost-efficient application of effective therapies. The Tumor Marker Utility Grading System (TMUGS) is a framework that establishes an agenda for evaluating the clinical utility of tumor markers, and describes five levels of evidence as

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"categories that define the quality of data available on which the utility score is based". These levels range from the weak level V evidence, derived from case reports and clinical examples, to the strongest level I evidence, "the definitive demonstration of clinical utility, obtained by a single, high-powered, prospective, randomized, controlled trial or from a meta-analysis or overview of multiple, well-designed studies", passing through intermediate degrees of evidence in levels II–IV (2).

Many studies have illustrated the potential of liquid biopsy as tumor marker, to determine the genomic profile of cancer patients, monitor treatment responses and quantify minimal residual disease, and assess the onset of therapy resistance. The concept of liquid biopsy has gradually evolved reaching nowadays a wider field of application from the initial idea of "leukemic phase of solid tumors", used to indicate the presence of epithelial cells of putative tumor origin in the peripheral blood of cancer patients (3). Circulating tumor cells (CTCs) were described for the first time in 1869 by Ashworth, who documented the presence of cells "identical with those of the cancer itself" in the blood of a metastatic cancer patient (4). In 2001, Pachmann *et al.* combined laser scanning cytometry with immunomagnetic bead enrichment to detect and quantify rare tumor cells in peripheral blood and bone marrow, describing this method as the most sensitive to "enable extensive investigation of the seeding behaviour of tumours" (5). Three years later, the association of CTC number and patient outcome was demonstrated in metastatic breast cancer using the CellSearch® platform (Janssen Diagnostics, LLC, Raritan, NJ, USA), a semi-automated system that immunomagnetically enriches epithelial cell adhesion molecule (EpCAM)-positive CTCs and enumerates them as (EpCAM+)CK+DAPI+CD45– cells (6).

Since then, substances that could be extracted from blood, serum and plasma have been included in the concept of liquid biopsy, such as circulating cell-free nucleic acids (DNA, RNA or microRNAs), and exosomes (7). Today, the term also incorporates several other biological materials obtained from almost all body fluids (urine, pleural effusion, ascites, etc.) (8).

In the field of CTCs, despite the great number of technologies developed in order to enrich, isolate and characterize these metastatic seeds of tumors, only the CellSearch® technology has demonstrated the clinical validity of CTC quantification (level I evidence) as a prognostic factor—in terms of progression-free survival (PFS) and OS—for metastatic breast cancer, by a pooled analysis of individual patient data published in 2014 (9).

The CellSearch® CTC Kit has been cleared by the Food and Drug Administration (FDA) [2008] for detection and enumeration of CTCs of epithelial origin in whole blood of metastatic colon, prostate and breast cancers. For these pathologies, the EpCAM+ CTC number strongly correlates to prognosis: a cut-off value of CTC/7.5 mL of peripheral blood has been validated, and patients whose CTC number is above this cut-off are at high risk of progression and short OS (6,10-12). Since 2015, the CTC level has been included in the American Society of Clinical Oncology (ASCO) and in the National Comprehensive Cancer Network (NCCN) guidelines for its prognostic value in metastatic breast cancer, consistently with the analyses published by Bidard and colleagues (9). However, its predictive value and clinical utility are still under discussion, and ongoing clinical trials will define the possibility to use this assay for treatment decision together with currently used diagnostic methods.

More in detail, current limits to an extensive use of CTC assays in the clinical setting include: (I) the lack of a consensus about the features that are necessary and sufficient to define an event as CTC; (II) the poor ability to track tumor cells undergoing epithelial-to-mesenchymal-transition (EMT), responsible for the loss of epithelial markers in favor of the mesenchymal ones; (III) the sensitivity of the techniques with a limit of detection of CTCs in no more than 50% of metastatic patients (13); (IV) the lack of a comprehensive genomic characterization of the circulating compartment together with a reliable assessment of its heterogeneity.

In summary, a major limit to the use of CTC assays and, more in general, of liquid biopsy into clinical practice is the lack of a consensus on standard operating procedures (SOPs) for its assessment. Indeed, robust, reproducible and shared procedures are mandatory for reaching the clinical validation of the best assays to isolate and/or characterize the tumor burden in peripheral blood, and for definitively proving their individual or complementary use as companion diagnostic.

This review will summarize current developments on liquid biopsy in non-small cell lung cancer (NSCLC), addressing the limits for the use of CTCs/ctDNA in the clinical practice, and analyzing the solutions adopted to overcome such limits, on the road towards the clinical validation.

Liquid biopsy in lung cancer

Cancer is a leading cause of death worldwide: there were

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8.8 million cancer deaths in 2015, most of which due to lung cancer (http://www.who.int/mediacentre/factsheets/fs297/en/). About 80% of lung cancers are classified as NSCLC, 15% are small cell lung cancer (SCLC), and 5% are other histological variants (14). Current therapeutic options are chemotherapy and drug therapy directed against specific molecular targets, mainly epidermal growth factor receptor (EGFR) missense mutations and deletions, and anaplastic lymphoma kinase (ALK) rearrangements. However, response rates are generally modest and patients are still exposed to side effects of both cytotoxic agents and targeted therapy (15). Moreover, most patients are still treated with palliative chemotherapy, due to advanced disease at diagnosis.

Targeted therapies rely on the availability of tumor biopsies for molecular profiling at diagnosis and relapse; these procedures might be invasive, and cause sample bias due to tumor heterogeneity, within the primary tumor and among different metastatic sites, resulting in single-site biopsy being not representative of the complete molecular profile of the systemic disease (16). Thus, current diagnostic tools are hampered by tumor heterogeneity and evolution giving rise to resistance. Furthermore, re-biopsy is not feasible to longitudinally monitor treatment response and resistance development, due to suboptimal quality and poor material recovered by bronchoscopy and invasiveness of the procedure.

Liquid biopsy has emerged as a minimal invasive approach to respond to the urgent need for real time monitoring, stratification, and personalized optimized treatment. In principle, a liquid biopsy could provide the genetic landscape of all cancerous lesions, detecting genomic alterations sensitive to targeted therapy or associated with treatment resistance. Moreover, it would guarantee the prognostic/predictive biomarkers evaluation in patients for whom biopsies are inaccessible or difficult to repeat.

CTCs and circulating tumor microemboli (CTM)

CTCs are cancer cells that detach from the primary tumor or metastatic sites, enter the bloodstream and might develop into further metastases. They are found in around 50% of patients affected by metastatic epithelial tumors (13). CTCs are extremely rare events, occurring at an estimated frequency of one against 10^6–10^7 leukocytes (17). They can travel in the bloodstream as single cells or cell clusters, called Circulating Tumor Microemboli (CTM) (18). CTM consist of tumor cells, fibroblasts, leukocytes, endothelial cells, pericytes and platelets held together by the expression of cell adhesion proteins such as plakoglobin. They are considered to play an important role in the development of metastasis, because tumor cells within clusters are protected by both the presence of neighboring cells and the expression of adhesion molecules, and are thus more able to escape from immune surveillance and reach distant organs (19,20). Hou *et al.* compared the molecular characteristics of CTM and solitary CTCs found in SCLC patients: in CTM, apoptotic cells were absent and tumor cells were not proliferating, supporting the hypothesis that they might have a survival advantage and be more resistant to chemotherapy than individual proliferating CTCs (21).

Cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA)

cfDNA has recently emerged as a promising diagnostic tool for cancer patients: cfDNA originates from both normal and tumor cells that undergo apoptosis or necrosis, and from macrophages that phagocytize necrotic cells. A higher level of cfDNA has been detected in cancer patients when compared to healthy subjects, and in later-stage versus early-stage tumors (16). Notably, cfDNA level can increase also in physiological (intense physical activity) and para-physiological conditions (pregnancy), and daily oscillations are observed due to the circadian rhythm (22). Moreover, specific non-tumor scenarios, such as inflammation, end-stage renal failure, stroke, myocardial infarction, surgery, and trauma are associated with high levels of cfDNA (23).

ctDNA is the cfDNA portion specifically derived from apoptotic, necrotic or living tumor cells that actively release DNA in the circulation. Several studies investigated the association of ctDNA levels and clinical outcome in lung cancer patients. However, since the absolute ctDNA amount is poorly significant as a diagnostic tool, the attention has focused on real-time monitoring of tumor-associated mutations for tracking the response to target therapies and/or the early onset of drug resistance (7). In this context, recent techniques such as digital PCR (dPCR) and next generation sequencing (NGS) have overcome the limit of sensitivity of traditional methods, such as Sanger sequencing, allowing the detection of ctDNA even if it represents a poor fraction of the total cfDNA (<1.0%) (23).

Exosomes

Exosomes are cell-derived vesicles of 40–100 nm in

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diameter that are released by several cytotypes into the extracellular space. They contain proteins, lipids, DNA, mRNA, microRNAs and other non-coding RNAs, and are thought to be involved in cell communication and metastasis (24,25). There is growing interest towards these nano-sized vesicles because the exosomal molecules are protected from RNase/proteinase-dependent degradation, and can be stably detected in the circulating compartment, making them ideal biomarkers for several clinical applications (26). Interestingly, Taverna *et al.* isolated plasma exosomes from a chemo naive 70-year-old patient with stage IV NSCLC, harboring an EGFR activating mutation (27). A panel of 8 miRNAs, with a documented role in NSCLC cell lines (28), was analyzed by real-time PCR, and their abnormal expression documented, consistently with previous findings (29). This study revealed that exosomes-derived microRNAs might constitute novel biomarkers in NSCLC diagnosis and prognosis.

Current limits for lung CTC detection

The EpCAM matter—EMT CTCs

Since CTCs are rare and dispersed into a huge amount of blood cells, their detection/isolation usually requires an initial enrichment step in order to increase their concentration. Enrichment methods are based on their biological characteristics (expression of cell surface protein markers, usually EpCAM for positive selection and/or CD45 for negative selection) or physical properties (size, density, deformability, or electric charges). CTCs can then be detected by immunologic, molecular or functional assays. *Table 1* reports some of the most commonly used technologies for CTCs enrichment and characterization in lung cancer.

EpCAM is a membrane glycoprotein highly expressed in the majority of carcinomas, and therefore of potential use as a diagnostic and prognostic marker for a variety of cancers (51). The EpCAM-based technologies allow the detection of CTCs from most epithelial solid tumors. Among those, the CellSearch® system has offered robustness, reproducibility and cost effectiveness, providing the first *in vitro* diagnostic (IVD) FDA-approved CTC assay for monitoring breast, prostate and colon cancer patients (6,11,12). The system uses ferrofluid particles coated with anti-EpCAM antibodies to capture CTCs. The enriched cells are then stained with fluorescent antibodies anti-panCytokeratin (CK 8, 18, 19; epithelial marker), anti-CD45 (leukocytes), and DAPI for nuclear labeling. A cell is classified as CTC if EpCAM+/CK+/CD45–, with a minimum size of 4 μm × 4 μm, and a DAPI+ nucleus occupying at least 50% of the CK+ cytoplasm. Speaking about NSCLC, the prognostic value of CTCs detected by the CellSearch® system was first reported by Krebs and colleagues in a cohort of 101 patients, with previously untreated stage III/IV NSCLC. Their study demonstrated that patients with 5 or more CTCs/7.5 mL of blood had a worse prognosis compared with those with less than 5 CTCs (33).

Several studies argued that CTCs in NSCLC may be underestimated when using EpCAM-dependent methods, due to EpCAM downregulation during EMT, an important mechanism for tumor invasion and metastasis. This hypothesis was first explored by Farace *et al.* using in parallel the CellSearch® system and the Isolation by Size of Epithelial Tumor cells (ISET®) technology in 60 patients with metastatic breast (n=20), prostate (n=20) and NSCLC (n=20). ISET® is an EpCAM-independent, filtration-based system, allowing CTCs isolation followed by cytological characterization (*Table 1*). The study highlighted an important difference in CTC numbers between the two techniques depending mostly on tumor type, and particularly prominent in metastatic NSCLC patients (49). These results were confirmed also by Krebs *et al.* in 40 chemo-naive advanced NSCLC patients. In this latter study, CTM were detected in 38% of patients with stage III/IV NSCLC by ISET®, whereas they could not be finely characterized by the standard CellSearch® assay, suggesting the need of a customized *ad hoc* assay for CTM detection with this platform. Furthermore, the analysis of EpCAM expression by immunohistochemistry on ISET®-isolated CTCs/CTM of 9 selected patients showed that all detected cells were negative for this marker, consistently with the lack of CTC detection by CellSearch® in the majority of this subgroup of patients (18).

Even if the EpCAM-independent methods, have demonstrated to detect a higher number of CTCs in NSCLC patients, larger well-designed clinical studies are needed to validate their biological and clinical significance. At this regard, the Foundation for the National Institutes of Health (FNIH) Biomarkers Consortium states that "the mere comparison of two assays metrics/unit blood" is not sufficient to assert that a new assay is more sensitive than a gold standard assay; instead, it is mandatory to determine if the new assay can still retain or even improve the robust capacity of the gold standard to discriminate the outcome of patients who are positive versus those who are negative for

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Table 1 CTCs technologies in lung cancer

Technology	CTC enrichment	CTC detection	Comments	References
Label-dependent systems				
AdnaTest	Immunomagnetic beads anti-EpCAM and/or other tumor-associated markers	RT-PCR for cancer specific targets	CTC number not quantifiable	Tewes (30) Andreopoulou (31) Hanssen (32)
CellSearch® System	Anti-EpCAM coated immunomagnetic beads	Immunocytochemistry: CK8,18,19+CD45-DAPI+ CTCs	Semi-automated platform. FDA-cleared for metastatic breast, prostate and colorectal cancer	Cristofanilli (6) Allard (13) Krebs (33)
CTC-Chip	Microposts array coated with anti-EpCAM Abs	Immunofluorescence: CK+CD45-DAPI+ CTCs	Microfluidic technology. Unlabeled cells are available for subsequent molecular analyses	Nagrath (34) Sequist (35) Zhang (36)
GILUPI Cell Collector®	EpCAM-coated medical wire	Immunofluorescence: EpCAM+ and/or CK+, CD45-DAPI+ CTCs and morphology	Process large volumes of blood for in vivo CTCs collection	Saucedo-Zeni (37) Gorges (38)
HB-CTC (Herringbone-Chip)	EpCAM Ab-coated surface combined with high-throughput microfluidics	Immunofluorescence: CD45-DAPI+ CTCs and tumor associated targets	Increased interactions CTCs-chip surface due to microvortices. Allows CTM detection	Stott (39)
MACS® (Magnetic Activated Cell Sorting System)	Paramagnetic beads with anti-EpCAM, panCK, HER2/neu, or CD45 Abs. Separation by high-gradient magnetic fields	–	Cells available for subsequent analyses	Miltenyi (40) Mayo (41)
RosetteSep™ (Human Circulating Epithelial Tumor Cells Enrichment Cocktail)	Negative selection (i.e., anti-CD45) combined with gradient density centrifugation	–	Highly purified, unlabeled cells for downstream analyses	He (42) Hodgkinson (43) Morrow (44)
Label-independent systems				
ClearCell® FX1 System	Size-based selection using centrifugal force	–	Isolates unlabeled cells. Requires RBC lysis. Small CTCs may escape detection	Lim (45) Tan (46)
DEPArray™ System	Cells trapped in DEP cages and sorted by morphological parameters	–	Isolates unlabeled, purified single cells. Requires pre-enrichment	Fabbri (47) Hodgkinson (43)
ISET® (Isolation by Size of Epithelial Tumor cells)	Filtration based on cell size and deformability	Epithelial and/or vimentin immunocytochemistry; cytomorphological detection	Filtration-based technology. Isolates CTM; small CTCs may escape detection	Hofman (48) Farace (49) Krebs (18) Ilie (50)

Ab, antibody; CK, cytokeratin; CTM, circulating tumor microemboli; CTCs, circulating tumor cells; DEP, dielectrophoretic; FDA, Food and Drug Administration; HER2/neu, human epidermal growth factor receptor 2; RBC, red blood cells; RT-PCR, reverse transcriptase-polymerase chain reaction.

the assay (52).

This item was first addressed by De Wit *et al.* that used an innovative method to investigate the presence of both EpCAM+ and EpCAMlow/– CTCs in the same patient sample, by the collection of the blood discarded by the CellSearch®, after immunomagnetic enrichment of EpCAM+ CTCs, followed by filtration of the EpCAMlow/– cells, and immunofluorescent detection. The study confirmed that the CellSearch® system is very efficient in recovering cells with relatively high EpCAM expression, but less effective with cells showing low/no EpCAM expression. A large portion of cells discarded by the CellSearch® system could be recovered by filtration, however, the efficacy depends on the size of the cells and

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smaller tumor cells may be missed using this approach. The study confirmed the association between EpCAM+ CTCs and poor patient outcome, whereas no association was found between EpCAMlow/– cells and outcome (53). The small cohort analyzed (27 metastatic lung cancer patients), however, limits the conclusions of this study and highlights the need of a multicenter pooled analysis to get a better picture of the EpCAMlow/– CTCs role in lung cancer.

Concerning EpCAM-negative CTCs and patient outcome, a more recent study from Pailler *et al.* evaluated the predictive value of ALK-aberrant (rearranged or with copy number gain, CNG) CTC number in 39 metastatic ALK-positive NSCLC patients treated with crizotinib. The group combined immunofluorescence staining (DAPI/CD45) and filter adapted-fluorescence in situ hybridization [FA-FISH; (54)] to perform ALK FISH on ISET®-enriched CTCs (at baseline and at an early treatment time-point). No association was found between baseline ALK-aberrant CTC number and PFS/OS (median ALK-rearranged CTCs 14/3 mL, range 1-119; median ALK-CNG CTCs 12/3 mL, range 1–53). Conversely, during treatment there was a significant association between a decreased ALK-CNG CTC number and longer PFS (median ALK-rearranged CTCs 13/3 mL, range 3–60; median ALK-CNG CTCs 15/3 mL, range 2–177). Although this study highlights the potential use of dynamic changes of ALK-CNG CTC number for monitoring treatment efficacy in ALK-positive NSCLC, its results still apply to a small cohort of patients, and reflect the lack of a consensus on CTC definition (i.e., no epithelial/mesenchymal additional marker for CTC detection by ISET®). Moreover, the parallel CellSearch® analyses revealed a median CTC number of 0/7.5 mL detected both at baseline and during treatment (range 0–713 and 0–544, respectively), thus the EpCAM-positive CTC fraction, that so far has been demonstrated to correlate with NSCLC patient outcome, was not further associated to clinical data in this study (55).

Notably, EMT CTCs and CTM could be only two of the factors affecting CTC detection: in fact, CTCs expressing a different CK-pattern from the most commonly detected in epithelial-dependent assays (CKs 4–6, 8, 10, 13, 18, 19) have been documented in lung cancer (53). Secondly, apoptotic CTCs have been assessed in carcinoma patients, suggesting that the detection of viable cells could be crucial to identify the subset of CTCs most likely involved in the metastatic process (56). All together, these findings underline the urgency of a profound molecular characterization of the different CTC subpopulations identified in NSCLC.

The volume issue: CTC assay sensitivity

Currently, CTCs are detected in around 50% of patients affected by epithelial tumors with distant metastases (13), with the CellSearch® assay identifying 20–40% of CTC positive metastatic NSCLC patients (32,33,48,57). In order to increase CTC detection in several cancer types, lung cancer included, other solutions have been investigated. In particular, recent efforts have focused on sample volume (58) as a crucial technical issue for assays sensitivity, moving towards the processing of large volumes of blood to increase CTC detection, particularly in early stage and/or low CTC rate tumors, such as NSCLC (37).

The Gilupi CellCollector® is a CE-approved device developed in order to screen large volumes of blood (*Table 1*). The medical device consists of a stainless steel wire with a functionalized surface composed of a hydrogel matrix tip coated with anti-EpCAM antibodies. The device is inserted into the cubital vein of patients for 30 minutes and captures CTCs *in vivo*. Gilupi CellCollector® obtained promising results on CTCs isolated from several cancer patients with a detection rate of around 70% in both early and late cancer stages (http://www.gilupi.com/cellcollector.html).

Two groups reported the *in vivo* use of this medical wire in lung cancer: the first study was conducted in 12 NSCLC patients and 29 healthy volunteers. After removal of the device from the patient's arm vein, bound CTCs were detected by immunofluorescence, as described in *Table 1*. CTCs were observed in all patients with a median of 16 CTCs (range 2–515), whereas no CTC was detectable in healthy volunteers (37). The second report was a prospective, blinded, single-center clinical study, including 50 patients with newly diagnosed and locally advanced/metastatic lung cancer (48 NSCLC patients) and 10 healthy individuals. Patients underwent two subsequent device applications during the same visit, to assess the reproducibility of measurements with the medical wire, before and after 12 weeks of therapy, and CellSearch® analysis was performed in parallel from 7.5 mL of blood collected immediately before the wire application. CTCs captured by the device were stained and counted similarly to the previous study [(37) and *Table 1*]. The results of the two subsequent CTC analyses performed in both visits detected >1 CTC in 58% of analyzed wires (median 5 CTCs, range 1–56), with 78% of CTC-positive patients at the first pre-therapeutic visit, and 72% at the second post-therapeutic. The incidence of CTC-positive patients was higher if determined by the wire compared with the

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CellSearch® technology (27% positive patients, range 1–300 cells), showing a median of 3 more CTCs per patient with the wire. CTC positive wires from 2 patients were further characterized by chip-based dPCR for specific KRAS and EGFR mutations of the primary tumor (38). The advantages of the CellCollector® technology is that the hydrogel matrix can be coated with antibodies directed against different surface markers in order to isolate CTCs with low EpCAM expression, and, moreover, that collection occurs *in vivo*. A limit, however, is that the volume of blood that gets in contact with the device during the 30 minutes can vary around 1.5–3 L (counts reported as CTC/30 min) (37), probably affecting the quantitative data among different patients or time points in the same patient. Nevertheless, the study from Gorges *et al.* reported reproducible results between two subsequent device applications during the same visit of the patient, underlining the utility of this method to increase CTC capture rates. Furthermore, they demonstrated the feasibility to perform molecular characterization of device-isolated CTCs that can provide important information on therapeutic targets or resistance mechanisms in cancer patients (38).

Recently, Fischer and colleagues introduced the idea that diagnostic leukapheresis (DLA), could be used to isolate a large number of CTCs even in non-metastatic cancer patients. DLA is a standard method frequently used in the clinical setting to isolate mononuclear cells (MNCs) from peripheral blood for various applications including stem cell harvest. In their study, they suggest that DLA would enable CTCs collection together with MNCs, as they have similar density. CTCs were detected in more than 90% of non-metastatic breast cancer patients by a CellSearch® test adapted to DLA, and a correlation was found between CTC numbers and anatomic disease spread. This study also combined DLA to genomic single-cell profiling and suggested their use in order to improve both the prediction of therapy response and the monitoring of early systemic cancer (59).

High throughput microfluidics: improving CTC recovery

Since CTCs are extremely rare events, occurring at an estimated frequency of one against 10^6–10^7 leukocytes (17), the use of high throughput microfluidic-based platforms has emerged in the field of CTC technologies, with the aim to improve their recovery efficiency in the huge amount of contaminating leukocytes. These platforms isolate unlabeled cells—down to a single-cell level—with minimum sample pre-analytical processing, allowing their downstream molecular profiling, *ex vivo* and *in vivo* applications (cell cultures, drug testing, xenografts).

The ClearCell® FX system has been recently developed as an EpCAM-independent microfluidic-based enrichment method (*Table 1*). This technology takes advantage of the microfluidic CTC-Chip (*Table 1*) to isolate unlabeled CTCs based on size, deformability and inertia. Tan *et al.* used this system to isolate CTCs from 27 NSCLC patients (12 wild-type, 14 ALK-rearranged and 1 with unknown ALK status) and test them for ALK translocation by FISH, similarly to what Pailler *et al.* reported in ISET®-isolated CTCs (46,54). The results of both studies provide evidence of highly concordant ALK rearrangement patterns between CTCs and tumors, suggesting a CTC utility as a diagnostic tool, and for monitoring resistance during follow-up.

In the context of *ex vivo* studies, Zhang *et al.* implemented a co-culturing model to expand CTCs captured from 19 early stage lung cancer patients. They used a microfluidic device similar to the CTC-Chip (*Table 1*) to isolate CTCs (68% CTC positive patients, range 1–11/mL, median 3 CTCs), and further expand them *in situ*, on the chip, setting up an ideal tumor microenvironment (73% expansion efficiency). This *ex vivo* technique for culturing CTCs opened a new scenario for enriching early stage CTCs, in order to understand their role in the metastatic process (36). Nevertheless, the bias of an *in vitro* selective pressure that might select the more aggressive CTC clones must be considered.

Exploring the metastatic potential of lung CTCs

Regarding *in vivo* studies, Hodgkinson *et al.* demonstrated the tumorigenicity of CTCs enriched from 6 extensive-stage chemotherapy-naive SCLC patients by RosetteSep™ technology (*Table 1*), and injected in immunocompromised mice. Interestingly, the CTC number detected by CellSearch® in the paired blood samples of those giving rise to CTC-Derived eXplant (CDX) were all >400/7.5 mL, whereas lower CTC numbers did not generate CDX (range 20–1,625; 67% CDX efficiency, 4/6 patients). Moreover, CellSearch®-enriched CTCs from the blood samples of two patients with the highest numbers, giving CDX (1,625 and 1,376 CTCs/7.5 mL, respectively), were recovered using the DEPArray™ system: single CTCs and groups of cells were isolated and genomic profile was performed showing a strong correlation between isolated CTCs and their corresponding CDX, analyzed in parallel (43).

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In a more recent case study reported from the same group, the authors obtained a CDX from the blood of a 48-year-old NSCLC patient. Three blood samples were drawn at each visit, before chemotherapy and after completion of brain radiotherapy (after the first cycle of chemotherapy the patient had brain progression), and used for CTC enrichment before mice implantation, CellSearch® and ISET® analyses. A CDX was obtained only from the post-radiotherapy, but not from the baseline sample (4 CTC/7.5 mL). CTC analyses of the post-radiotherapy sample matched to the CDX, showed 0 CTCs/7.5 mL by the CellSearch®, and >150 CTCs/mL by ISET® (CK+ and/or Vimentin+ CD45– CD144–). 77% of CTCs were positive for the mesenchymal marker Vimentin. This study reported for the first time a CDX generated from a NSCLC patient without detectable EpCAM+CK+ CTCs, supporting the idea that not only EpCAM+ (43) but also mesenchymal CTCs have a tumor initiating potential in NSCLC (44). In order to further investigate this hypothesis, it would be mandatory to assess the efficiency of CDXs generated from NSCLC patients with no/few CellSearch® detectable CTCs within larger cohorts of patients.

In these and other *in vivo* studies, one major limit for an application of xenografts in co-clinical trials focused on direct patient treatment is the large number of CTCs necessary to obtain a CDX, which is hardly detected in NSCLC patients. To date, Rossi *et al.* obtained mice xenografts from EpCAM-enriched CTCs (CellSearch® Profile Kit) of 5 prostate and 2 breast cancer patients with around 50 CellSearch® detected CTCs in the matched blood sample (2 prostate patients had 51 and 66 CTCs/7.5 mL, respectively; CTC range 51–2,866; 100% engraftment efficiency in peripheral blood, 8/8 mice), thus supporting the role of EpCAM+ CTCs in initiating metastases and opening a new scenario in the understanding of the metastatic potential of CTCs (60).

Genomic characterization of the circulating compartment

NSCLC is one of the most clonal heterogeneous tumors, with a variety of driver-mutations characteristic of different patient subsets. The majority of these genomic alterations include EGFR, ALK, and KRAS genes. Mutations in EGFR gene are commonly localized in the tyrosine kinase domain. Patients whose primitive tumor results positive for EGFR mutations are normally treated with tyrosine kinase inhibitors (TKIs: erlotinib, gefitinib, 1st-generation; afatinib, 2nd-generation) but, unfortunately, often develop resistance within 9–12 months. In many cases, resistance lies on the establishment of secondary mutations such as the EGFR-T790M, responsible for a reduced TKI binding to its ligand (61).

Another target for NSCLC therapy is the EML4-ALK-fusion oncogene, currently assessed on tumor biopsies or fine-needle aspirations. ALK translocation is reported in 2–7% of advanced NSCLC, and is a negative prognostic marker. However, its detection by FISH analyses allows for targeted therapy with crizotinib (1st-generation) and ceritinib (2nd-generation), which is more effective than standard chemotherapy (61).

These tumor "druggable" mutations have been largely investigated in the circulating compartment of NSCLC patients, also thanks to recent highly sensitive methods, such as NGS and dPCR, which allow the detection of low abundance mutations in the context of a huge amount of non-tumor circulating cells/nucleic acids. In principle, the concordance of genomic alterations between tumor biopsies and CTCs/ctDNA has been demonstrated by several reports, both for EGFR mutations (62-64) and ALK translocations (50,54,65), leading to the inclusion of CTCs/ctDNA in clinical trials as prognostic/predictive biomarkers. The results of these studies suggest that monitoring EGFR mutations or EML4-ALK rearrangements in the circulating compartment can reveal therapy resistance also prior to radiological progression evidence, thus guiding the therapeutic decision in the patient clinical course (62,65). In this context, Marchetti and colleagues conducted a feasibility study to analyze EGFR mutations by NGS in CellSearch®-enriched CTCs of 37 locally advanced/metastatic NSCLC patients (stage IIIB/IV) recruited within the TRIGGER study. 15 of the 37 patients analyzed were CTC positive by standard CellSearch® analyses (41% positive patients, range 1–29 cells). Moreover, in 33 cases, "potential neoplastic elements" were detected including cells not fulfilling all CellSearch® criteria (the "suspicious objects" defined by the instrument manual), and isolated or clustered large naked nuclei with irregular shape. All the 37 CellSearch®-derived samples were subjected to mutational analyses by NGS, and results were compared to the mutational status of the primary tumor as detected by Sanger sequencing (all EGFR positive). The study supported the coupling of the CellSearch® system with ultra-deep sequencing as a powerful method for following EGFR mutations in CTCs (31/37 CTC-enriched samples were EGFR positive), and highlighted a 94% concordance of the mutation type

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between primary tumor and the circulating compartment. Interestingly, in four CTC-enriched samples, the study disclosed multiple EGFR mutations in CTCs by NGS, when the corresponding primary tumor had only a single activating mutation detected by Sanger sequencing. However, the possibility that those multiple mutations could be present also in minor clones of the primary tumor, but being undetectable by Sanger sequencing, could not be excluded because NGS was not performed on the primary tumor (66). Nevertheless, this and other studies document a genetic heterogeneity for EGFR mutations in lung cancer, with cases of multiple EGFR mutations in tumor tissues [detected by NGS, (67)], and CTCs (62). Moreover, a different mutational status between primary tumor and CTCs/ctDNA was also reported elsewhere (32,68). Notably, these studies also indicate the mandatory need to use methods that are more sensitive in the analysis of tumor biopsies at diagnosis, rather than Sanger sequencing.

Tumor heterogeneity might be responsible for progression and pharmacological resistance. In this context, the use of untargeted approaches (array comparative genomic hybridization, whole genome sequencing/exome sequencing) has the advantage, besides the high sensitivity, to allow a wider screening and enable the identification of unknown alterations that might be responsible for a specific cell phenotype (stem-cell, EMT, invasiveness) and/or therapeutic response. In this sense, coupling enrichment and isolation of single CTCs with the most recent developed high-sensitivity assays has revealed a great potential to simultaneously assess tumor heterogeneity and monitor tumor-associated genetic markers (43,47,69).

The long road towards clinical utility

To date, the role of CTCs as a prognostic and predictive biomarker in lung cancer is still puzzling. Several studies associate CTC number to OS and/or PFS of metastatic NSCLC patients (32,33,48,57), meanwhile others do not find these associations (70,71). Many small trials have also demonstrated the association between CTC count and therapy efficacy, comparing the count before and after therapy (38,72-74), or during target therapy, in order to detect the onset of resistance mutations (50,54,62,66,75). Even though most data suggest the utility of CTCs as biomarkers for assessing prognosis and monitoring therapy response in NSCLC, these assays are still not used in routine clinical practice, mainly due to the lack of standardized procedures allowing reliable and reproducible results that could be compared among different cohorts of patients.

This review has addressed the technology issues that contribute to the poor ability to track tumor cells in the blood of NSCLC patients, thus hampering the extensive use of CTCs in the clinical setting. The limit of EpCAM-based CTC technologies, excluding EMT cells from being isolated and quantified, has been discussed. EMT CTCs are documented in NSCLC (76), and low numbers of CTCs are observed with epithelial marker-dependent methods (13,33). Indeed, methods based on physical properties (48,49,53) or PCR-based methods (32), usually detect higher numbers of CTCs in lung cancer. However, so far, the FDA-cleared CellSearch® assay, enriching and counting EpCAM+ CTCs (*Table 1*) has proved to identify 20-40% of CTC positive metastatic NSCLC patients (32,33,48,57). Moreover, a CTC positive status has been associated with a positive lymph node using this test (32,77). On the other side, the EpCAMlow/– fraction of CTCs alone has not been shown to correlate with patient outcome (53). Consistently, in other types of metastatic tumors, mesenchymal CTCs alone have been only weakly associated with OS and PFS (78). All together, these data suggest that a complementary dual technology, detecting both epithelial and mesenchymal CTCs might allow to obtain a much clear picture of the potential of these cells in predicting patient outcome (18).

Two great issues in CTC detection are assay sensitivity and recovery efficiency. At this regard, we described the processing of large volumes of blood to increase CTC detection, in early non-metastatic patients (59) and in metastatic NSCLC (37,38), conditions in which CTCs occur at very low rate. This approach has revealed the potential to greatly improve CTC sensitivity, allowing recovery of higher cell number for downstream molecular analyses (59), and increasing detection rate up to 70% CTC positive patients in lung cancer (38). In addition, the use of high throughput microfluidic-based platforms has greatly improved CTC recovery in the huge amount of contaminating leukocytes [1 CTC against 10^6–10^7 leukocytes (17)], achieving the isolation of pure, unlabeled CTCs (36,43,46). The combination of these technologies allowing rescue of a great number of pure CTCs down to a single cell level, together with the recently developed highly sensitive genomic profile techniques (NGS and dPCR among others), has promising results in terms of personalized management of the NSCLC patients (38).

Similar to CTCs, also in the context of ctDNA, its low abundance, compared to the huge amount of constitutive cfDNA, is a major limit of detection. Also in this case, the

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availability of highly sensitive tests, has permitted to overcome this issue and deepen analyses of this circulating compartment. Since several other physiological and pathological features can influence its absolute amount, cfDNA is a weak diagnostic tool in the absence of specific tumor-associated mutations (8). For this reason, the clinical interest on ctDNA has focused mainly on real-time monitoring of specific tumor-associated mutations as prognostic biomarker for tracking the response to target therapies and/or the early onset of drug resistance, with promising results in terms of patient follow-up (79).

Together these findings suggest that combined molecular analyses of CTCs and ctDNA in blood samples may be useful to monitor the dynamic changes in the mutation profile that occur during therapy as well as the heterogeneity that emerges as a result of the therapeutic selective pressure. Observations arising from these tumor evolution mechanisms during therapy could be used to define target treatments that suppress the clones responsible of drug resistance before they become clinically relevant.

For all the reasons here described, current studies, focused on reaching a consensus on liquid biopsy utility in NSCLC, are multicenter pooled analyses with larger cohorts of patients, longer observation periods, and CTC/ctDNA molecular characterization according to standardized procedures. In this context, the CANCER ID consortium has started in 2015 (35 partners from 13 countries) with the aim to establish standardized protocols, in order to obtain reliable, reproducible tests, allowing the genomic and molecular characterization of the circulating compartment together with the cross-comparison between different technologies. The final goal is to clinically validate these blood-based biomarkers in NSCLC and breast cancer patients (https://www.cancer-id.eu/).

The long road towards clinical utility of liquid biopsy is probably much harder than it was expected when the "leukemic phase of solid tumors" was first described, but, along the way, the circulating compartment is already revealing its huge potential in the era of precision medicine and personalized care of cancer patients.

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Footnote

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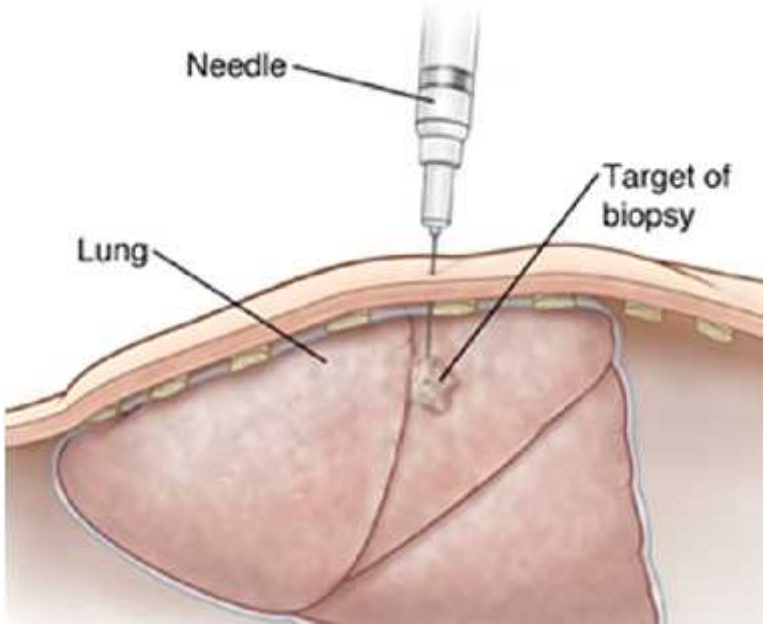
Liquid Biopsy in Cancer Diagnosis & Monitoring

Reza Shahsiah, MD, Pathologist

March 2019

Opening Question

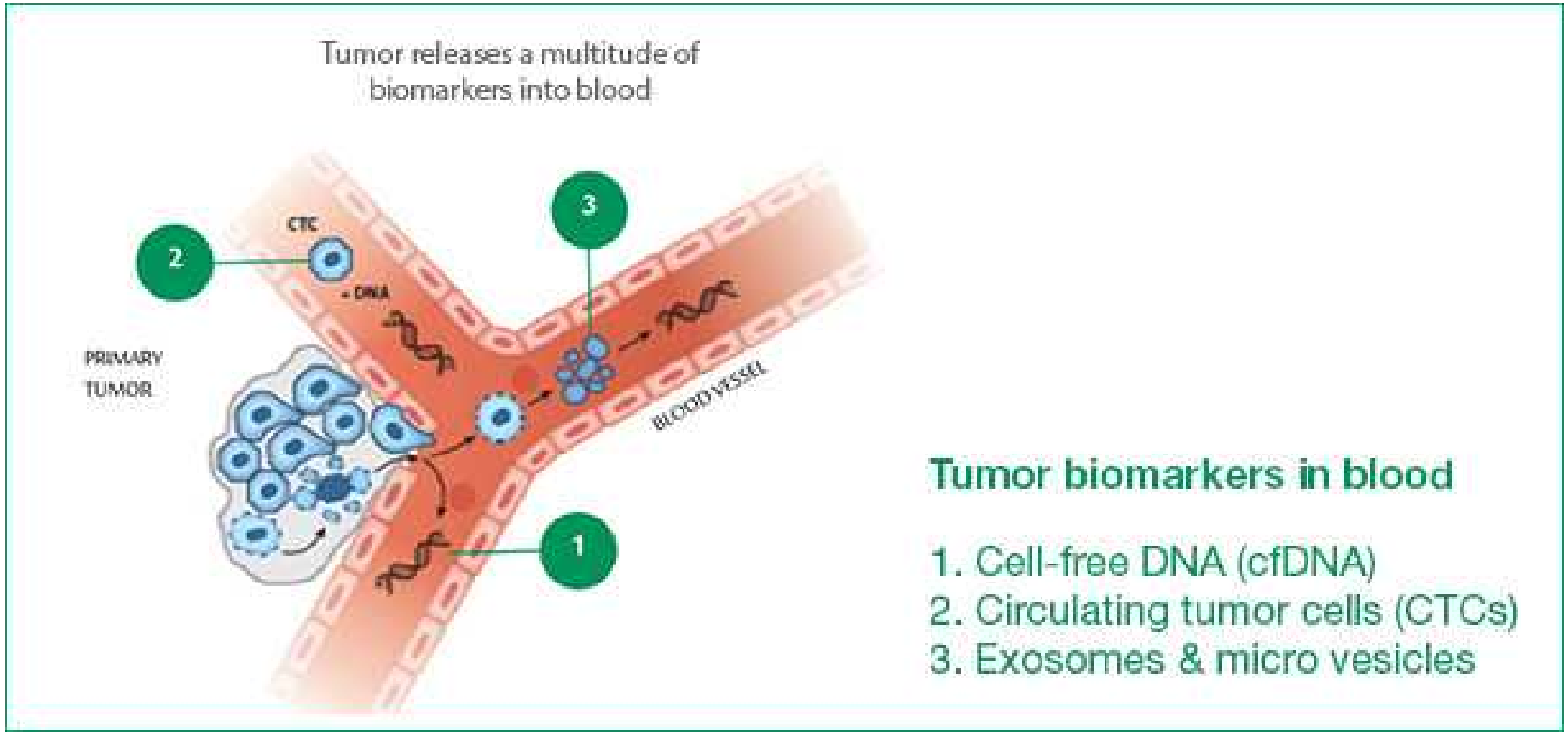
Can liquid biopsy or plasma biopsy replace tissue biopsy?



Definitions

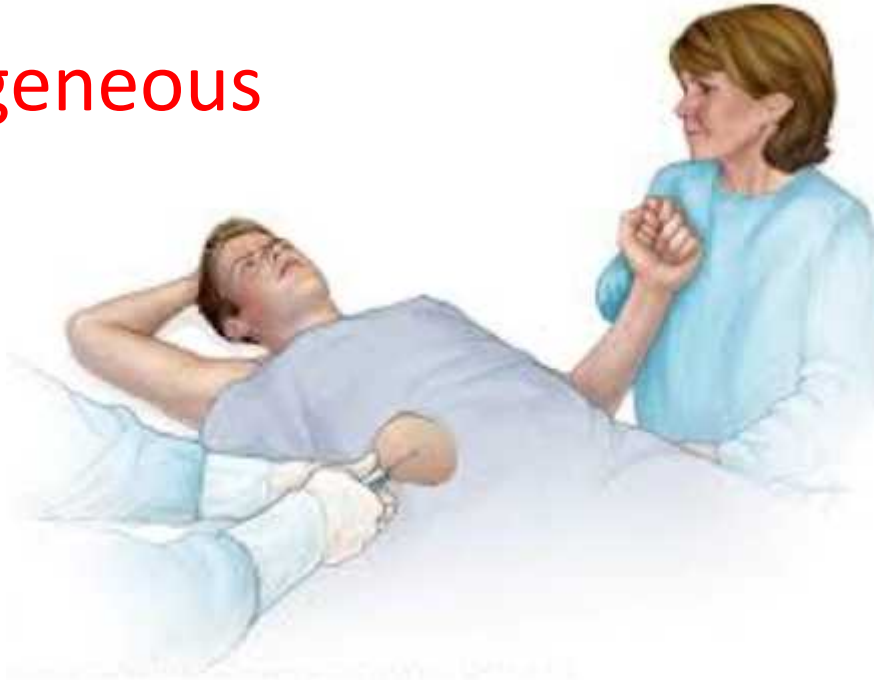
The term “liquid biopsy” qualifies different potential approaches for the detection of biomarkers produced by cancer in circulating blood.

Tumor Products

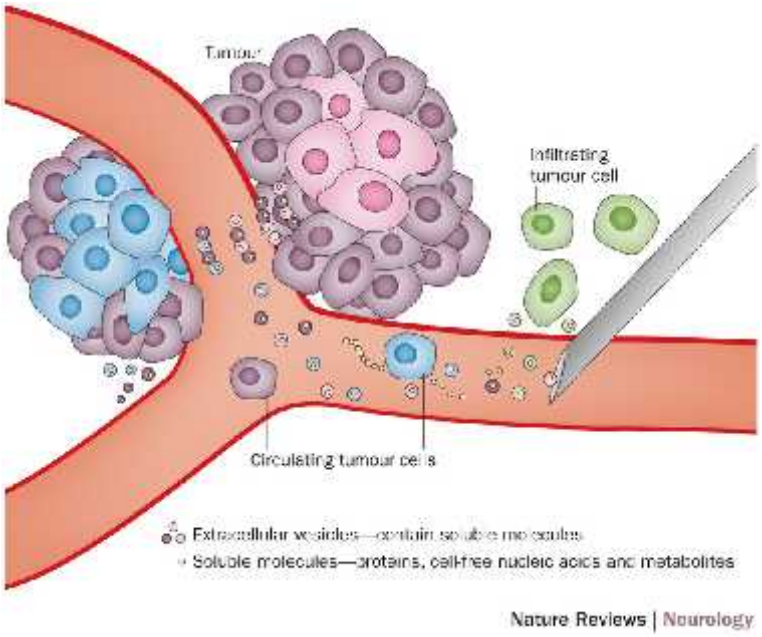


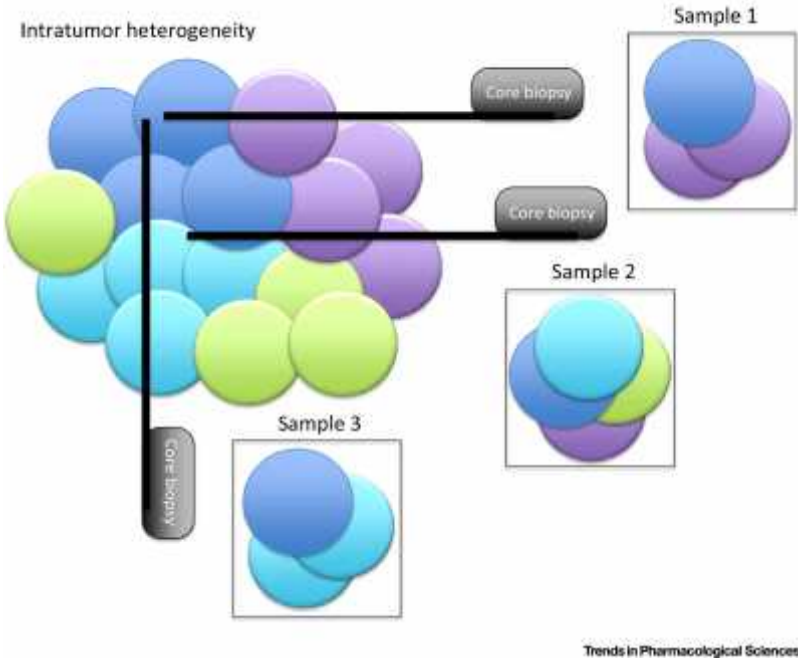
Disadvantages of Tissue biopsy

- Its hard to obtain adequate tumor tissue
- Tissue biopsy is invasive
- Tumors are heterogeneous



Tumor Heterogeneity





Circulating Tumor Cell

- Circulating tumor cell that CTCs are formed by cell detachment from the primary tumor mass
- Detection of CTCs is technically challenging since it is estimated that as few as one CTC can be found per 10^6–10^7 peripheral blood mononuclear cells

Circulating Tumor DNA

Cell free DNA (cfDNA) originates through cell necrosis or apoptosis. Peripheral cfDNA exists in low concentrations of less than 100 ng/mL—and only a fraction of this total is actually Circulating Tumor DNA (ctDNA).

The fraction of ctDNA that is tumor derived in cancer patients ranges from <0.1% to >30%

Challenges

- Body cells release cfDNA, and CTCs into the bloodstream, but the majority of cfDNA, or nucleated cells is often not of cancerous origin, making it sometimes difficult to detect tumor-derived alterations
- Before CTCs or endosomes can be used in NSCLC, clinical validation and further standardization is needed

Validation

Many standard techniques for mutation detection are not useful for ctDNA analyses due to an insufficient sensitivity. Since ctDNA often represents a small percentage of the total cfDNA, somatic mutations coming from the tumor can be present at allele fractions as low as 0.01%. Highly sensitive methodologies, or variations of preexisting methodologies, have been developed in order to detect low abundance mutations in cfDNA.

Application of Liquid Biopsy

1. Screening and early detection
2. Making the Diagnosis
3. Determining the molecular profile (needed for planning chemotherapy) in treatment-naïve patients
4. Detecting the emergence of resistance (i.e EGFR T790M mutation) in patients who are under therapy

FDA approved Method

The Roche Cobas EGFR Mutation Test v2 is the only ctDNA assay that is currently approved by the US Food and Drug Administration (FDA) for the detection of *EGFR*-sensitizing and resistance mutations in NSCLC. This platform was originally approved by the FDA for the detection of *EGFR*-sensitizing mutations in tissue. Recently, this approval has been extended to include plasma genotyping for both *EGFR*-sensitizing mutations and the T790M resistance mutation.

Disadvantage

If the liquid biopsy is negative, then tissue biopsy is recommended if feasible because ctDNA may be less than the detection limit of the assay and most sensitive assays may not detect a mutation or a tumor is not shedding ctDNA

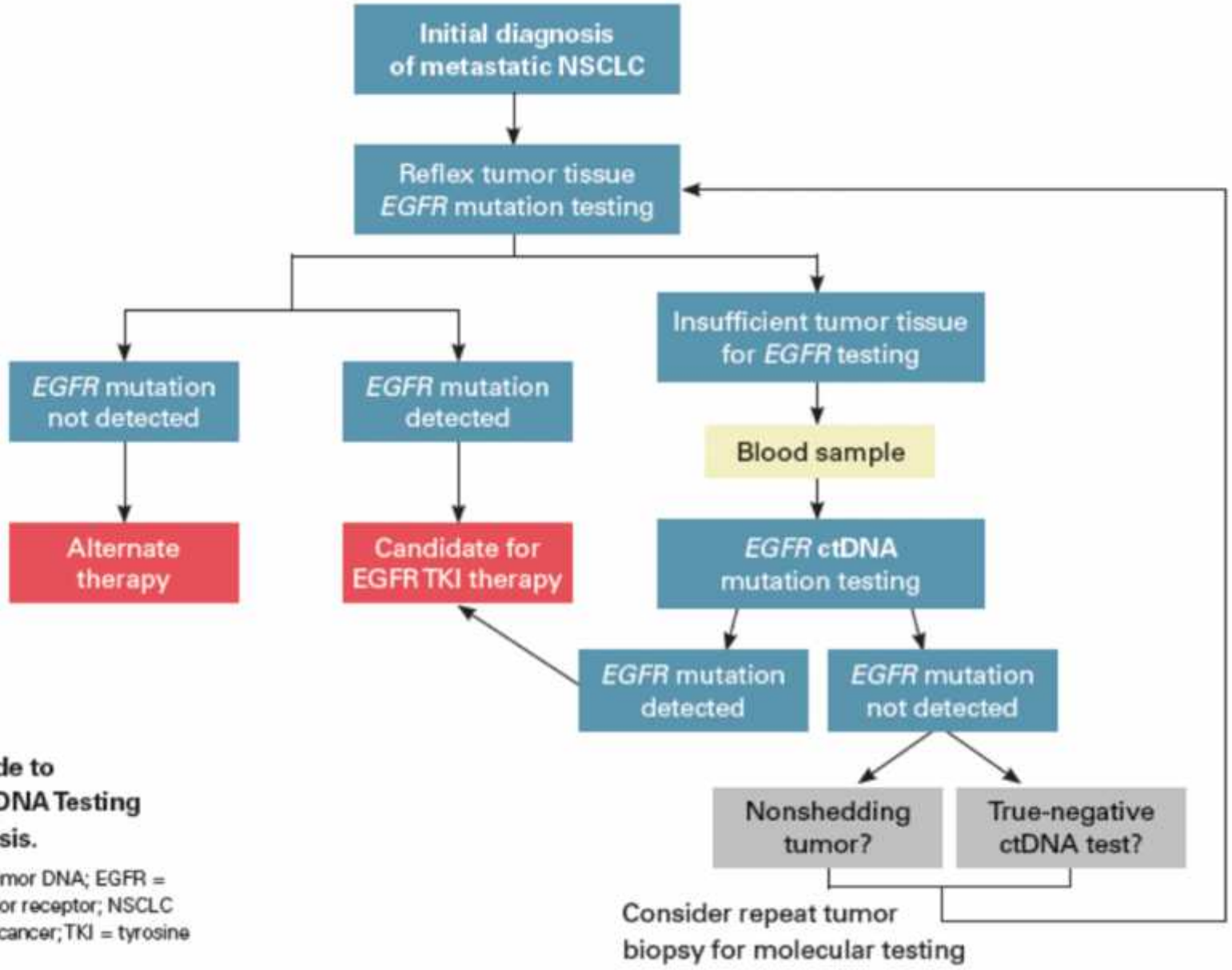


Figure A. Guide to Utilization of ctDNA Testing at Initial Diagnosis.

ctDNA = circulating tumor DNA; EGFR = epidermal growth factor receptor; NSCLC = non–small-cell lung cancer; TKI = tyrosine kinase inhibitor.

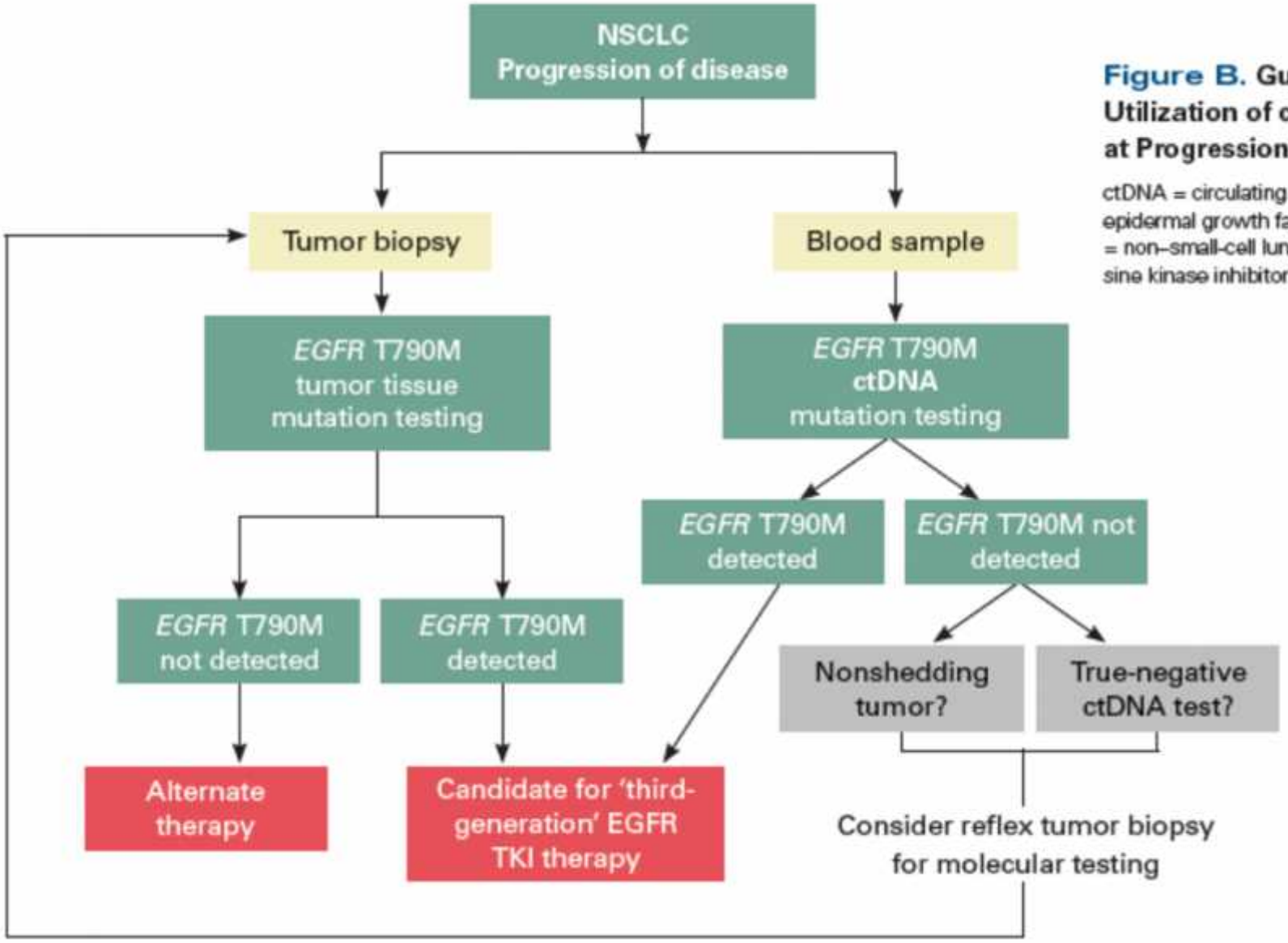


Figure B. Guide to Utilization of ctDNA Testing at Progression of Disease.

ctDNA = circulating tumor DNA; EGFR = epidermal growth factor receptor; NSCLC = non–small-cell lung cancer; TKI = tyrosine kinase inhibitor.

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Review Article

Liquid biopsy based biomarkers in non-small cell lung cancer for diagnosis and treatment monitoring

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Abstract: Advances in the knowledge of the biology of non-small cell lung cancer (NSCLC) have revealed molecular information used for systemic cancer therapy targeting metastatic disease, with an important impact on patients overall survival (OS) and quality of life. However, a biopsy of overt metastases is an invasive procedure limited to certain locations and not easily acceptable in the clinic. Moreover, a single biopsy cannot reflect the clonal heterogeneity of the tumor. The analysis of peripheral blood samples of cancer patients represents a new source of cancer-derived material, known as liquid biopsy, and its components can be obtained from almost all body fluids. These components have shown to reflect characteristics of the status of both the primary and metastatic diseases, helping the clinicians to move towards a personalized medicine. The present review focuses on the liquid biopsy components: circulating tumor cells (CTCS), circulating free DNA (cfDNA), exosomes and tumor-educated platelets (TEP); the isolation technologies used and their potential use for non-invasive screening, early diagnosis, prognosis, response to treatment and real time monitoring of the disease, in NSCLC patients.

Keywords: Liquid biopsy; non-small cell lung cancer (NSCLC); circulating tumor DNA (ctDNA); circulating tumor cells (CTCs); exosomes; tumor-educated platelets (TEP)

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Introduction

Lung cancer is the leading cause of cancer death worldwide, with non-small cell lung cancer (NSCLC) accounting for the majority of cases. It is the most frequent cancer both in terms of incidence (1.2 million new cases, 12.3% of the global total) as well as with respect to mortality, approximately 1.2 million deaths per year, which is equivalent to 17.8% of the world total (1).

Patients with NSCLC experience poor overall survival (OS) (2) due to the presence of metastatic diseases that are not adequately detected and therefore many patients are subjected to aggressive localized treatments, such as surgery or radiotherapy (RT), when in reality they present advanced distant disease.

Tissue biopsy has always been the standard diagnostic procedure for tumors and also to provide enough material for genotyping, which can assist in the targeted therapies of cancers. However, tissue biopsy-based cancer diagnostic procedures have limitations in their assessment of cancer development, prognosis and genotyping, due to tumor heterogeneity and evolution (3). As a matter of fact, there is growing evidence for intratumor heterogeneity indicating that single-site biopsies fall short of revealing the complete genomic landscape of a tumor (4).

A liquid biopsy, or blood sample, can provide the genetic landscape of all cancerous lesions (primary and metastases). In addition, it offers the opportunity to systematically monitor cancer disease as the quantification of circulating

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tumor DNA (ctDNA) has been shown to correlate with tumor burden (5). Circulating free DNA (cfDNA), exosomal RNA as well as circulating tumor cells (CTCs) or tumor-educated platelets (TEP) offer the potential for non-invasive screening, diagnosis, prognosis, and measuring response to treatment (6-8).

The practice of liquid biopsy as a diagnostic and prognostic tool in lung cancer patients is gradually becoming an appealing approach in the clinical routine practice, since it is noninvasive and can be easily repeated (3). In particular, this approach allows real-time monitoring of the patient during treatment, as well as the detection of different genomic alterations potentially sensitive to targeted therapy or associated with treatment resistance. It also allows rapid access to biomarker assessment in vulnerable lung cancer patients for whom solid biopsies are inaccessible or difficult to repeat. This attractive approach could promote a change in therapy, even before detection of tumor progression or relapse (5).

This Review will explore how tumor-associated genetic alterations detectable in the blood can be used in the clinic for diagnosis, assessment of prognosis, early detection of disease recurrence, and as surrogates for traditional biopsies with the purpose of predicting response to treatments and the emergence of acquired resistance.

Liquid biopsy

The term "liquid biopsy" qualifies different potential approaches for the detection of biomarkers found in circulating blood in cancer patients. Liquid biopsy analysis is a rapidly expanding field in translational cancer research and might be useful at different points of the diagnostic/therapeutic course of cancer patients, such as early diagnosis, estimation of the risk for metastatic relapse or metastatic progression (prognostic information), stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms (predictive information), and understanding metastasis development in cancer patients. Different liquid-derived materials can be employed, such as CTCS, ctDNA, exosomes, circulating RNA or microRNAs (9). They may be obtained from almost all body fluids (blood, serum, plasma, urine, pleural effusion, ascites, etc.) (10) and are likely to contain a wider presentation of genomic data from multiple metastatic sites, whereas mutations present in a single biopsy or minor subclone may be missed. In addition, the information obtained from liquid biopsies is allowing an increase into the knowledge of the pathophysiology of lung cancer and into the process of metastatic dissemination. .

Molecular profiling studies performed on tumor biopsies of NSCLC have identified genetic alterations that predict tumor responses to targeted therapies such as protein tyrosine kinase inhibitors (TKI). With an even larger repertoire of TKI entering the clinic, screening tumors for genomic aberrations is increasingly important (11). Detection of these driver mutations, such as EGFR mutations, in cfDNA or CTCs, has allowed to use targeted therapies avoiding invasive procedures and to identify resistance mechanisms for TKIs, while quantification of these mutations has helped to monitor tumor burden and dynamics (12,13). However, the mutational analysis of cfDNA demonstrated a significantly better sensitivity when compared with CTCs, establishing cfDNA as the best source of material for mutation detection (14).

In addition, the biological properties of mRNA content of exosomes and platelets have also contributed to provide a comprehensive real-time picture of the tumor burden, improving the application of genetic profiling of tumor associated RNA and DNA derived from biofluids and the understanding of cancer as a dynamic disease.

CTCs

CTCs are formed by cell detachment from the primary tumor mass, determining the migration of tumor cells to secondary sites via the lymphatic and blood system. The presence of CTCs has been demonstrated in the blood of patients with various solid tumors and it has been associated with poor outcome in metastatic NSCLC patients as well as in other tumors (10,15). Although CTCs were first described in 1869, their possible role the in the clinical setting since has progressively gained attention (16).

Detection of CTCs is technically challenging since it is estimated that as few as one CTCs can be found per 10^6–10^7 peripheral blood mononuclear cells in patients with advanced solid tumors, with less quantity of cells in patients with early-stage diseases. Due to this low concentration of CTCs, its detection and characterization require highly sensitive and specific methods, which consist in a combination of different strategies (17).

CTCs enrichment is either based on biological or physical properties (18). Biological properties include those approaches targeting epithelial specific surface markers like epithelial cell adhesion molecule (EpCAM) for positive selection (e.g., CellSearch™, microfluidic devices or the

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nanodetector) or CD45 for negative depletion (19,20).

The CellSearch™ (Veridex LLC) is the only approved methodology by the U.S. Food and Drug Administration (FDA). It utilizes EpCAM-coated magnetic beads to isolate CTCs but has shown a limited detection efficiency in several types of carcinomas. In metastatic NSCLC disease, Krebs *et al*. reported only 32% of patients having ≥2 CTCs before chemotherapy treatment (21).

The development of a unique microfluidic platform, the "CTCs-chip", has been demonstrated to be capable of efficient and selective separation of viable CTCs from peripheral whole blood samples. The chip is based on the interaction of target CTCs with EpCAM-coated microposts under precisely controlled laminar flow conditions, without requisite pre-labelling or processing of sample. This technology successfully identified CTCs in almost 100% samples analyzed of patients with metastatic lung, prostate, pancreatic, breast and colon cancer patient, with an average purity of 52% in NSCLC patients (22).

Despite all the advantages mentioned above, some tumor cell subpopulations can escape EpCAM-based isolation technologies, as some cells may undergo epithelial-mesenchymal transition (23). In consequence, other technologies based on non-immunological properties have been used for CTCs isolation. Physical properties include size (e.g., ISET, isolation by size of epithelial tumor cells) or differential density (e.g., FICOLL, isolation by centrifugation steps) (21,24). Using ISET, Krebs *et al*. reported an 80% detection rate of CTCs in peripheral blood samples collected from 40 chemonaïve, stages IIIA to IV, NSCLC patients, using ISET, compared with 23% using CellSearch™. A subpopulation of CTCs isolated by ISET did not express epithelial markers and circulating tumor microemboli (clusters of ≥3 CTCs) were observed in 43% patients using ISET but were undetectable by CellSearch™. They concluded that both techniques should be performed together as this dual analysis allows more complete exploration of CTCs (25).

As CTCs technology has evolved rapidly during last years, several attempts have been performed for this technology to have the potential to be used in tracking the genomic evolution of tumors over time, and may have therapeutic implications in terms of its ability to detect functional events or resistant subclones while avoiding the need to conduct repeated biopsies. Several studies have attempted to establish CTCs counts as a diagnostic, prognostic and predictive tool in NSCLC patients.

A study published in 2013 (26), aimed to determine CTCs detection as a diagnosis marker of NSCLC. CTCs were labeled by a folate receptor (FR) conjugate, showing a sensitivity of 73.2% and a specificity of 84.1% for the diagnosis of NSCLC (67.2% sensitivity in stage I). Of note, FRs are highly expressed in a variety of tumors, particularly in ovarian and lung cancers, whereas most normal tissues express low to negligible levels. A novel FR-based CTCs analysis method had been developed based on these findings (27).

CTCs have long been considered to reflect tumor aggressiveness and have been investigated as a surrogate marker for tumor growth. At this respect, the decrease in the number of CTCs during the treatment has been associated with radiographic tumor response and an increase in the cell counts with tumor progression (9). In particular, the decrease or disappearance of CTCs following surgery has been correlated with improved clinical outcomes (28,29). A recent study (30) has prospectively analysed the kinetics of CTCs in blood samples of 56 patients obtained before surgery and one month after. The results showed that the presence of CTCs after surgery was significantly associated with early recurrence (P=0.018) and a shorter disease-free survival (DFS) (P=0.008).

Changes in CTCs count after RT have also been studied. Dorsey *et al*. (31) analyzed 30 patients with localized NSCLC undergoing radiation treatment. Using a telomerase-based detection assay, 65% of them were CTCs positive prior to treatment, but CTCs numbers significantly dropped after irradiation (9.1 *vs*. 0.6 CTCs/mL; P<0.001).

In metastatic NSCLC patients, a number of studies have reviewed the prognostic significance of baseline CTCs count, concluding that it is an independent negative prognostic factor that correlates with advanced stage a shorter progression-free survival (PFS) and OS (21). Using the CellSearch system, Krebs *et al*. (21) reported median OS of 8.1 *vs*. 4.3 months (P<0.001) and PFS of 6.8 *vs*. 2.4 months (P<0.001) for stage III–IV NSCLC patients when CTCs cut-offs of <5 *vs*. ≥5 were applied. In the multivariate analysis, the number of CTCs was the strongest predictor of OS (HR 7.92; 95% CI, 2.85–22; P<0.001) exceeding the traditional risk factors of stage and performance status. In another study including 46 patients with newly diagnosed or recurrent NSCLC, CTCs were measured at baseline and before every chemotherapy cycle in a subset of patients (n=23). A baseline CTCs count of more than eight prior to chemotherapy was a strong predictor of reduced PFS (P=0.018) and OS (P=0.026). However, no correlation was observed between CTCs count and tumor size after two chemotherapy cycles (32).

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Finally, several studies have provided evidence of an association between a decrease in CTCs counts and radiographic response by FDG-PET/CT or RECIST. In a phase II clinical trial of erlotinib and pertuzumab with 41 patients enrolled, higher baseline CTCs counts were associated with better response to treatment by RECIST (P=0.009), and decreased CTCs counts after treatment correlated with FDG-PET and RECIST responses (P=0.014 and P=0.019) and a longer PFS (P=0.05) (14). However, a more recent study failed to confirm this finding, probably because it included a heterogeneous cohort of patients with respect to stage and histology, which can confound interpretation of FDG uptake and CTCs analysis (33).

In all, these studies indicate that CTCs count might be useful as a prognostic marker in lung cancer patients and can be of helpful in therapy decision making, especially for the selection of those surgically rejected patients at a higher risk of relapse who might benefit from adjuvant therapies (5).

Biomarker detection in CTCs

The development of personalized medicine and targeted therapies has led to an increased interest in CTCs as a source of material for genetic analyses. The detection and characterization of CTCs represent a major opportunity as they reflect both the phenotypic and the genetic information of the primary tumors. Also, the analysis of CTCs allows real-time evaluation of metastasis and can show the molecular condition of the disease (10).

EGFR status has been examined in CTCs from advanced NSCLC patients, allowing the identification of sensitizing mutations and the T790M resistant mutation. Using the CellSearch System coupled with next-generation sequencing (NGS), Marchetti *et al*. (34) identified for the first time *EGFR* mutations in the CTCs of 84% of patients EGFR positive in matching tumor tissue. In another study (35), a novel assay based on real-time PCR and melting curve analysis was developed to detect activating *EGFR* mutations in blood cell fractions enriched in CTCs. Using this assay, EGFR-positive CTCs were detected in pretreatment blood samples from all eight *EGFR*-mutant lung cancer patients studied (35).

Despite good initial responses, all NSCLC patients carrying sensitive *EGFR* mutations eventually develop resistance to *EGFR*-TKIs. The T790M gatekeeper mutation is present in 40–60% of those patients at disease progression. The development of third-generation *EGFR* inhibitors capable of overcoming T790M-associated resistance has led to a need for noninvasive testing of the T790M mutation (36). In a recent study comparing tumor biopsies with simultaneously collected CTCs and ctDNA, the resistance-associated mutation was detected in 47% to 50% of patients respectively, with a concordance ranging from 57% to 74%. (37). In those patients where the paired biopsy was negative or inconclusive, CTCs and ctDNA-based assays together enabled genotyping in 35% of cases.

ALK rearrangements, which are tested in NSCLC for crizotinib treatment, have also been evaluated in CTCs using a filtration enrichment technique and filter-adapted fluorescent in situ hybridization (FA-FISH). All ALK-positive patients were found to have four or more ALK-rearranged CTCs per mL of blood (median, 9 CTCs/mL; range, 4–34 CTCs/mL). In contrast, 0 to 1 ALK-rearranged CTCs/mL were detected in ALK-negative patients (median, 1; range, 0–1). The assay not only enabled diagnostic testing but also monitoring of crizotinib treatment (38). Similarly, ROS1 rearrangements, another target for crizotinib-based therapy, have been detected using ISET and FA-FISH in the CTCs of 4 patients with positive paired tumors for ROS1-gene (39).

In spite of all these technological advances, CTCs are still not used in the routine clinical practice or as a source of material for biomarker analysis in advanced NSCLC Although FDA approved CellSearch as CTCs detection method in metastatic breast, prostate and colon cancer, it has not yet been approved in metastatic lung cancer patients. Several limitations such as the lack of epithelial biomarkers which hamper CTCs detection, the lack of validation in large multicenter studies to evaluate reproducibility, specificity and sensibility and the lack of clinical validation in different cohorts of lung cancer patients highlight the need of additional research in advanced NSCLC in order to implement its application in the clinical setting (5).

Circulating free tumor DNA

ctDNA has the potential to enable noninvasive diagnostic testing for personalized medicine since it can provide similar molecular information as invasive tumor biopsies. Currently, the evaluation of specific predictive biomarkers is mandatory for a proper treatment of advanced-stage NSCLC patients according to the molecular characterization of the disease. In some cases, this molecular profiling can be difficult due to a reduced availability of tumor tissue, and therefore ctDNA testing might be used as a surrogate.

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cfDNA is single- or double-stranded DNA (dsDNA) that exists in plasma or serum. Early studies showed that many cancer-associated molecular characteristics, such as single-nucleotide mutations, methylation changes and cancer-derived viral sequences can be found in the cfDNA. These findings were significant for the development of ctDNA detection technology. Nevertheless, ctDNA-based assays face several challenges. Body cells release cfDNA into the bloodstream, but the majority of cfDNA is often not of cancerous origin, making it sometimes difficult to detect tumor-derived alterations (9). Also, prior knowledge about particular mutations can be of help, but may be difficult to obtain. However, recent technological advances have overcome these limitations, making it possible to identify both genetic and epigenetic alterations in cfDNA of cancer patients (3).

The fraction of cfDNA that is tumor derived in cancer patients ranges from <0.1% to >30% of the total DNA, and depends on tumor burden, stage, cellular turnover, accessibility to circulation and factors affecting blood volume. The ctDNA released by tumor cells will carry the same genetic (somatic) alterations as the tumor itself and this genetic load can be detected and quantified. At this respect, the relative levels of ctDNA have been shown to correlate with tumor burden and response to therapy (40).

Technical developments have allowed the identification of low frequency alterations in cfDNA. These platforms include real-time quantitative PCR (qPCR); digital PCR (dPCR); beads, emulsion, amplification and magnetics (BEAMing); and NGS. The sensitivity of these techniques ranges from 15% to 0.01%, and one of the major obstacles for their clinical applicability is the lack of standardization (41).

Real time qPCR: real time qPCR has been widely used for the identification of genetic alterations and the quantification of nucleic acids. While qPCR is a standardized, relatively inexpensive technique, its precision for quantifying rare allele or genetic alterations present at very low levels is significantly lower compared to other techniques such as digital (dPCR) (42). However, most of the published studies adopted variants of this technology for the analysis of ctDNA in lung cancer.

Regarding detection of *EGFR* mutations in cfDNA using qPCR, Kimura *et al*. showed for the first time that plasma-derived *EGFR* genotype is predictive of subsequent clinical response to an *EGFR*-TKI. A 72.7% concordance was found in the cases analyzed by sequencing of the primary tumor and Scorpion ARMS assay of plasma, (43). Similarly, Mok *et al.* reported a concordance between tissue and cfDNA of 88%, with blood test sensitivity of 75% and a specificity of 96% using the cobas blood test, based on qPCR (44).

EGFR oncogenic mutations have also been analyzed in plasma cfDNA of patients included into different trials, such as a trial evaluating the combination of pertuzumab and erlotinib (14) or the IPASS trial (45), which compared gefitinib with carboplatin/paclitaxel. The sensitivity of ctDNA testing in identifying *EFGR* mutations compared with tumor tissue ranged from 43% to 100%, being able to identify *EGFR* mutations in patients with insufficient or unevaluable tissue.

dPCR: the unparalleled precision of this technology enables accurate measurements for the quantification of low frequency genetic alterations. In this methodology, target molecules are separated into a large number of partitions so that each partition receives a number of molecules (usually ranging from 0–2) theoretically following a Poisson distribution. In consequence, target molecules are amplified mostly on an individual basis and measurements rely on counting the total of positive partitions containing one or more target molecules and negative partitions where no amplification is detected. dPCR allows for the detection of mutated ctDNA in a high background of wild type cfDNA and for the quantification of small fold change differences. Several studies have assessed the feasibility of dPCR for biomarker testing and cancer monitoring and have evidenced that dPCR is indeed an adequate technology for such purpose (44-48).

BEAMing: this technique is based on a combination of emulsion dPCR with magnetic beads and flow cytometry for detection and quantification of mutant tumor DNA molecules (49). BEAMing is a highly sensitive but complex method to detect genetic mutations at very low levels (50). This methodology has been compared to mutation analysis of tumor tissue in a recent publication that tested 21 mutations in BRAF, *EGFR*, KRAS and PIK3CA in samples from different cancer types (13.8% of NSCLC). Results were concordant for archival tissue and plasma cfDNA in 91% cases for BRAF mutations, in 99% cases for *EGFR* mutations, in 83% cases for KRAS mutations and in 91% cases for PIK3CA mutations, demonstrating that BEAMing is feasible and highly concordant with tumor tissue analysis (51). Parallely, Taniguchi *et al*. used this technology for plasma testing of *EGFR* mutations (52) in 44 patients with *EGFR* mutant NSCLC (23 with progressive disease after TKI and 21 TKI-naïve). Activating mutations were detected in the cfDNA of 72.7% of the patients, while the T790M was found in 43.5% of the cases with a progressive disease.

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To assess the ability of different technologies to detect *EGFR* mutations in ctDNA, including T790M, a comparison of multiple platforms was undertaken (53). It included two non-digital (cobas™ *EGFR* mutation Test and therascreen™ *EGFR* amplification refractory mutation system assay) and two digital platforms (Droplet Digital ™ PCR and BEAMing digital PCR). This study used samples from a trial that investigated the safety and efficacy of AZD9291, a third-generation *EGFR*-TKI, in patients with a sensitizing mutation whose disease progressed after a TKI. For *EGFR*-TKI-sensitizing mutations, high sensitivity (78–100%) and specificity (93–100%) versus tissue was observed using the four platforms. Regarding the T790M mutation, the digital platforms outperformed the non-digital platforms. Subsequent assessment using 72 additional baseline plasma samples was conducted using the cobas *EGFR* mutation Test and BEAMing dPCR and using a tissue test result as a non-reference standard. For the T790M detection, the sensitivity and specificity were 73% and 67% for the first technology and 81% and 58%, respectively, with a concordance between the platforms >90%. Another recent publication comparing BEAMing and cobas test showed a higher concordance between BEAMing plasma and tumor EGFR results than between cobas *EGFR* plasma and tumor (82% for activating mutations and 73% for T790M using BEAMing *vs*. 73% and 64%, respectively, using cobas plasma test) (54).

NGS: the emerging of new drugs that target other genomic alterations than *EGFR* mutations have significantly increased the complexity of biomarker testing. In the context of a growing number of driver mutations to test, NGS presents itself as a suitable technology since it allows simultaneous detection of multiple alterations in a very efficient manner. However, the use of this technology might be limited by a modest sensitivity. Using deep sequencing as a detection system for *EGFR* mutation in the ctDNA of stage I–IV NSCLC patients, Uchida *et al*. reported a diagnostic sensitivity and specificity for exon 19 deletions of 50.9% and 98%, respectively; and for the L858R mutation of 51.9% and 94.1%,. Although overall sensitivity was 54.4% for all cases; it dropped to 22.2% in stages IA-IIIA, compared to 72.7% in stages IIIB–IV, highlighting that NGS might be restricted to advanced disease (55). Similarly, Couraud *et al*. used the IonTorrent Personal Genome Machine for the deep sequencing of the most relevant hotspot somatic mutations (*EGFR*, BRAF, KRAS, HER2, and PIK3CA) in tumor and plasma cfDNA of never-smoker NSCLC patients. In ctDNA, 50 mutations (36 *EGFR*, 5 HER2, 4 KRAS, 3 BRAF, and 2 PIK3CA) were identified. Sensitivity of the test was 58% and the estimated specificity was 87% (56). Because sensitivity appears to be compromised in early stages, several strategies to solve this issue are being tested. Newman *et al*. have developed an ultrasensitive method for quantifying ctDNA that can detect ctDNA in 100% of patients with stage II–IV NSCLC and 50% of patients with stage I, with 96% specificity for mutant allele fractions down to 0.02%. Researchers communicated that ctDNA levels correlated with tumor volume and distinguished between residual disease and treatment-related imaging changes. Measurement of ctDNA levels allowed for earlier response assessment compared to radiographic approaches, facilitating personalized cancer therapy (57). However, low quantities of ctDNA in blood and sequencing artifacts could limit the analytical sensitivity of this methodology. To overcome these limitations, the same groups have recently developed other technological approaches for improved detection of ctDNA (58).

Finally, two meta-analyses exploring the diagnostic value of cfDNA for the detection of *EGFR* mutation status have been published (59,60). Luo *et al.* [2014] reviewed 20 studies involving 2012 cases to assess the diagnostic performance of cfDNA compared with tumor tissue and concluded that detection of *EGFR* mutations in cfDNA has an adequate diagnostic accuracy. Likewise, Qiu *et al.* [2015] analysed 27 studies involving 3,110 patients and reported effectiveness similar to Luo *et al.*, with overall sensitivity of 62%, specificity of 96% and diagnostic odds ratio of 38.270.

Exosomes

Exosomes are vesicles of endocytic origin with a diameter of 40–100 nm that transfer information to the target cells (including proteins, DNA, mRNA, as well as non-coding RNAs) through at least three mechanisms: fusion with the plasma membrane, receptor-ligand interaction or endocytosis by phagocytosis (61). Thakur *et al*. provided also evidence that tumor-derived exosomes carry dsDNA, showing that exosomal DNA could reflect the mutational status of parental tumor cells (62).

Exosomes are critically involved in tumor initiation, growth, progression, metastasis, and drug resistance by transferring oncogenic proteins and nucleic acids. The role of exosomes in modulating the pathways that lead to the development of resistance to both RT and chemotherapy is an emerging area of intense research. Therefore, exosomes and their load could be biomarkers with value in diagnosis,

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prognosis and prediction of therapeutic responses (63).

Exosomes have been isolated and characterized from distinct cells under normal and stressed conditions. Several methods have been used for exosome isolation including ultracentrifugation, combined with sucrose gradient, and the immune-bead isolation [e.g., magnetic activated cell sorting (MACS)]. In addition, different commercial kits are available for their isolation and purification. Transmission electron microscopy (TEM), Western blot, and FACS are frequently used to characterize the isolated exosomes based on their biochemical properties, such as morphology, size or exosomal markers. However, there are not accurate methods to determine the concentration of exosomes and researchers have to rely on inaccurate measurements of protein concentration or nanoparticle tracking analysis. Quantitative RT-PCR, nucleic acid sequencing, Western blot, or ELISA has been used for exosome RNA and protein identification (64).

In patients with NSCLC, the circulating levels of tumor exosomes, exosomal small RNA and specific exosomal miRNA have been tested in order to validate them as a potential marker for diagnosis and prognosis in patients with adenocarcinoma (65).

Rabinowits *et al*. (66) reported significant differences in circulating exosomes and miRNA concentrations between lung adenocarcinoma patients and a control group. In addition, the similarity found between the circulating exosomal miRNA and the tumor-derived miRNA patterns led the authors to suggest that circulating exosomal miRNA might be useful as a screening test for lung adenocarcinoma. Similarly, another recent work reported a model based on microRNAs derived from circulating exosomes capable to discriminate between lung adenocarcinoma and granuloma (67).

Finally, the identification of large rearrangements in liquid biopsies such as the EML4-ALK translocation, that determines eligibility for treatment with FDA-approved ALK-kinase inhibitors, remains challenging. It appears reasonable to approach this issue by detecting the EML4-ALK fusion transcript. To this aim, the RNA protected in vesicles as the exosomal RNA appears to be a promising source for this kind of analyses and some diagnostic tests designed to identify the predictive biomarker EML4-ALK in liquid biopsy are based on EML4-ALK fusion transcripts detection using exosomal RNA (68).

TEPs

Blood platelets have recently emerged as important players in cancer development and responses to tumor growth. The confrontation of these platelets with tumor cells via transfer of tumor-associated biomolecules has been named "education". This interaction induces, among other processes, specific splicing of pre-mRNAs (69). In addition, platelets can ingest circulating RNA (70). Taken together, it appears that TEP could be a suitable source of material for liquid biopsy-based analyses. A recent publication by Best *et al*. (71) developed a classifier based on RNA-Seq of TEPs. Across six primary tumor types (NSCLC, colorectal carcinoma, glioblastoma, pancreatic cancer, hepatobiliary cancer, and breast cancer), the location of the primary tumor was correctly identified with 71% accuracy. Moreover, they identified several genetic alterations or biomarkers that could guide the use of drugs targeting those mutations. In addition to exosomes, TEPs seem to be an adequate source for fusion gene identification in liquid biopsies. Nilsson *et al*. have examined the use of RT-PCR to detect EML4-ALK rearrangements in platelets and plasma compared with matched tissue biopsies. RT-PCR demonstrated 65% sensitivity and 100% specificity for the detection of EML4-ALK rearrangements in platelets. Intriguingly, in a subset of 29 patients treated with crizotinib, the detection of these rearrangements in platelets was found to be correlated with lower PFS and OS. Authors also concluded that monitoring of EML4-ALK rearrangements in the platelets of one patient over a period of 30 months revealed crizotinib resistance two months prior to radiographic disease progression (72).

Collectively, these data suggest that TEP may provide a RNA BioSource for liquid biopsy based diagnosis (73).

Conclusions

Personalized medicine in oncology relies on the customization of healthcare using molecular analyses. In this context, diagnostic testing is used to select appropriate and optimal therapies based on a patient's cancer genome and to obtain molecular biomarkers in an easily accessible, minimally invasive way in order to follow the molecular profile of a patient's tumor longitudinally. The clinical application of liquid biopsy in NSCLC is progressively proving a pivotal tool for screening and early detection of cancer, real-time monitoring of therapy or risk of relapse (prognosis) and identification of therapeutic targets and resistance mechanisms.

ctDNA and CTCs in NSCLC patients are being intensively investigated, as they have proven to provide a

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dynamic picture of molecular disease changes and can be used to identify molecular alterations emerging during the course of treatment in a noninvasive manner. It is becoming increasingly clear that metastasis and tumors are extremely heterogeneous, and therefore each stage should be treated as a different condition. It has also been recently demonstrated that plasma ctDNA is suitable for monitoring treatment response and also for the early detection of acquired resistance mechanisms (e.g., T790M in NSCLC) (74). These and other studies are laying the foundations for the development of cfDNA as a new and non-invasive source of material that can be used routinely when testing for predictive biomarker for targeted therapies.

The value of CTCs analysis in lung cancer is currently mainly related to prognosis, but there is an increasing interest in the development of new techniques for molecular characterization of CTCs. It is not fully understood whether CTCSs are the only cause of metastasis, but it has been reported that their counts correlate with a poorer prognosis in lung cancer and in other tumor types, although there is still a need for clinical validation of these findings, particularly in lung cancer.

The information obtained from liquid biopsies, together with imaging analysis, replacing invasive tissue biopsies, will probably guide treatment decision and hopefully will improve the outcome for lung cancer patients, having also de potential for early diagnosis of tumors that are not yet visible on imaging.

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Footnote

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