Current and future applications of liquid biopsy in nonsmall cell lung cancer from early to advanced stages

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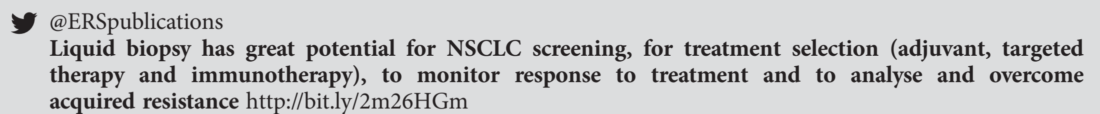

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Liquid biopsy has great potential for NSCLC screening, for treatment selection (adjuvant, targeted therapy and immunotherapy), to monitor response to treatment and to analyse and overcome acquired resistance http://bit.ly/2m26HGm

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ABSTRACT Liquid biopsy refers to the analysis of any tumour-derived material circulating in the blood or any other body fluid. This concept is particularly relevant in lung cancer as the tumour is often difficult to reach and may need an invasive and potentially harmful procedure. Moreover, the multitude of anticancer drugs and their sequential use underline the importance of conducting an iterative assessment of tumour biology. Liquid biopsies can noninvasively detect any targetable genomic alteration and guide corresponding targeted therapy, in addition to monitoring response to treatment and exploring the genetic changes at resistance, overcoming spatial and temporal heterogeneity. In this article, we review the available data in the field, which suggest the potential of liquid biopsy in the area of lung cancer, with a particular focus on cell-free DNA and circulating tumour cells. We discuss their respective applications in patient selection and monitoring through targeted therapy, as well as immune checkpoint inhibitors. The current data and future applications of liquid biopsy in the early stage setting are also investigated.

Liquid biopsy has the potential to help manage nonsmall cell lung cancer throughout all stages of lung cancer: screening, minimal residual disease detection to guide adjuvant treatment, early detection of relapse, systemic treatment initiation and monitoring of response (targeted or immune therapy), and resistance genotyping.

Introduction

Targeted therapies and, more recently, immune checkpoint inhibitors (ICIs), have transformed the treatment landscape of advanced nonsmall cell lung cancer (NSCLC). Response to these agents can be predicted by the use of companion biomarkers. There is currently a paradox between the need to obtain

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significant samples for multiple analyses for a growing number of molecular biomarkers and the development of minimally invasive or noninvasive techniques, resulting in small tissue samples with very small amounts of DNA. Cytological samples, such as endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), are often insufficient for a comprehensive molecular examination (10–20% of cases) [1, 2]. Moreover, a better understanding of the resistance to corresponding targeted therapies has led to a need for rapid, noninvasive, repeatable assays to assess and follow tumour biology through treatment.

It is within this context that there has been renewed interest in liquid biopsy, a concept which refers to any tumour-derived material circulating through the blood or any other bodily fluid [3]. Circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA), the most widely studied substrates in the field of NSCLC, have different advantages and disadvantages, and may be complementary. While tissue only offers a snapshot of the tumour at a given time and location (figure 1a), liquid biopsy has the potential to overcome both spatial and temporal tumour heterogeneity (figure 1b) and can noninvasively interrogate the molecular landscape of a tumour (taking into account different clones present within all metastatic sites) and can follow subclonal evolution through iterative blood draws (figure 1c).

This review focuses on the potential clinical applications of these two methods. Circulating exosomes, miRNA, RNA and tumour-educated platelets, which are not discussed in this article, are other appealing but immature approaches for tumour genotyping [4, 5].

Cell-free DNA

Terminology, physiopathology and biology

Cell-free DNA (cfDNA) corresponds to the cfDNA floating in the blood, but also other bodily fluids such as urine, pleural fluid, cerebrospinal fluid or cytology specimen-derived supernatant [6–9]. The first demonstration of circulating DNA was conducted in 1948 by Mandel and Metais [10]. These small DNA fragments (150–200 base pairs) can be passively released from apoptotic or necrotic cells or indirectly by tumour-associated macrophages [11, 12]. ctDNA tends to be more fragmented, with sizes ranging from 90 to 150 base pairs [13]. The amount of plasma DNA is much higher in patients with cancer (5–1500 ng·mL^{-1}) than in healthy patients (1–5 ng·mL^{-1}), with allelic frequency (AF; the fraction of mutated/wild type alleles) in blood being very variable in patients with cancer [14]. Nucleic acids can be found in blood bound to proteins, as oligo- or mono-nucleosomes, as a result of apoptosis, but also in membrane-bearing vesicles (exosomes, apoptotic bodies, microparticles) that are actively released by cells [15, 16]. An active secretion of tumour DNA into the bloodstream by the tumour has been demonstrated, albeit its pathophysiology has not yet been clearly deciphered. This release of nucleic acids might be used by the tumour to modify the profile of distant target cells and thus participate in the metastatic spread (genometastasis) [15, 17, 18].

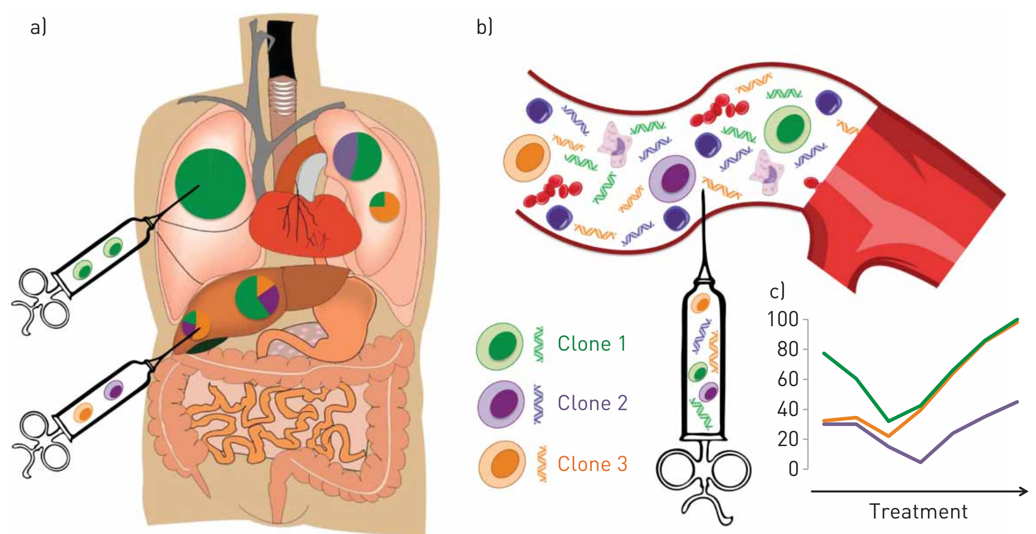

FIGURE 1 Liquid biopsy compared to tissue biopsy can capture both spatial (a *versus* b) and temporal (c) tumour heterogeneity and noninvasively follow the subclonal evolution of the disease through treatment.

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Genotyping platforms studied in lung cancer

Earlier studies have shown that cfDNA concentration is increased in patients with lung cancer [19], with a high level being correlated with a poor prognosis [20, 21]. Nevertheless, multiple inflammatory or infectious diseases can increase this level [22]. This problem can be avoided by targeting mutated tumour-specific DNA, which requires highly sensitive genotyping assays given that ctDNA only accounts for a very small proportion of the whole cfDNA, which is dominated by the germline, wild-type DNA compartment (the low mutant allelic fraction). Pre-selection based on the size of DNA fragments could increase AF and thus sensitivity and specificity of plasma genotyping [13]. Moreover, because it aims to be used as a screening test, and most targetable genotypes are of low prevalence in NSCLC, the specificity must be prioritised over sensitivity. Table 1 summarises the characteristics of the main plasma genotyping platforms available for lung cancer genotyping.

Targeted assays: EGFR *sensitising mutations and T790M screening*

Currently approved (US Food and Drug Administration (FDA) and European Medicines Agency) clinical use of cfDNA is limited to the cobas *EGFR* Mutation test v.2 CE-IVD (Roche, Basel, Switzerland) and Therascreen mutation kits (Qiagen, Hilden, Germany) for patients with NSCLC who are unable to undergo a tissue biopsy or with acquired resistance to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs). BEAMing (beads, emulsion, amplification and magnetics) [23] and digital-droplet PCR are other highly sensitive (0.01% AF) and quantitative approaches based on digital PCR [24]. Targeted assays can detect a known driver mutation (*EGFR*, *KRAS*, *BRAF*) [24–26], as well as the emergence of a pre-defined resistant clone (T790M) in blood weeks before clinical progression, and follow their variations through treatment [24, 27, 28].

Next-generation sequencing

The PCR-based assays described thus far only target one to three sites in a pre-defined gene, but fail to multiplex across several genes and to detect more complex genomic alterations, such as fusion genes. Next-generation sequencing (NGS) is a high-throughput sequencing method that can simultaneously interrogate variable areas of the genome and detect somatic mutations, including single-nucleotide variations (SNVs), copy number variations (CNVs), insertions/deletions or gene fusions.

Libraries preparation

The larger the genomic area an assay will target (coverage), the lower the depth (average number of reads obtained in a specific region of the genome) will be, which is a considerable criterion to take into account, since the lower the depth is, the more difficult it is to call a variant with confidence [29].

Whole-exome sequencing can simultaneously detect expected oncogenic drivers or mechanisms of resistance, and has the ability to discover/explore new molecular mechanisms of resistance. However, most clinically relevant fusions occur in noncoding regions and are missed by the whole-exome approach. Considering the trade-off between the depth and the amount of the genome covered, the use of panels of primers/probes targeting hotspots or exons of predetermined genes seems to be the most reasonable approach in the context of liquid biopsy, due to the very low fraction and amount of tumour DNA.

Hybrid capture NGS is a well-established approach in the liquid biopsy field [30, 31]. Predetermined DNA sequences are "captured" by hybridisation to biotinylated probes. The biotin is bound to streptavidin beads, allowing for the clearance of the remaining DNA. Because these technologies do not resort to prior amplification, they can reliably quantify copy number changes. For plasma genotyping, one disadvantage of hybrid capture is the inherent low input DNA, requiring platforms with a high depth of sequencing. This means there is a risk of sequencing error and false positives. For example, in a recent study, specificity, taking tissue as a reference, was 63.5%. The concordance rates between cfDNA and tissue were only 64.7% and 48.9% for pre-treatment and post-treatment specimens, respectively. Tumour heterogeneity and the time interval between tissue and blood sampling might partly explain these conflicting results, but since 100% of variants found at AF >1% in cfDNA were also found in tissue, sequencing errors might explain some of them [31]. Paweletz *et al.* [32] designed a bias-corrected NGS based on hybrid capture, but using single-primer amplification and tags for sample identification to reduce false positives.

Amplicon-based NGS enriches predefined sequences of interest by PCR amplification of exons/hotspots of specific genes [33]. This methodology appears to be particularly promising for cfDNA genotyping where the input DNA is low [34–36]. The use of molecular barcodes is needed to avoid false positives after PCR amplification: a PCR bias, present only on a very low fraction of reads carrying the same barcodes, will be distinguished from a true variant, whose number of reads will rise proportionally during amplification. However, PCR amplification can bias the observed allele frequency, but also the CNVs.

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TABLE 1 Characteristics of the major plasma genotyping assays available for advanced nonsmall cell lung cancer

	Principle	Coverage	Diagnostic accuracy (tissue reference)	Advantages	Pitfalls	Preferred indications
Targeted assays ddPCR BEAMing Cobas Therascreen	Genotyping of pre-defined hotspots, exons or complete genes of interest	1–7 hotspots of a gene (ddPCR) 7 hotspots (Therascreen) 42 mutations in four exons (Cobas)	Sensitivity 60–80% (*EGFR*) Specificity (*EGFR*) 96% Cobas, 97% BEAMing 100% ddPCR	Highly sensitive Highly specific Quantitative (except Cobas) Low turnaround time	Only targets pre-defined regions of interest: Doesn't cover all targetable alterations Doesn't capture a potentially subclonal resistance	Screening for pre-defined targetable mutations (*i.e. EGFR* activating mutation, *EGFR* resistance mutations (T790M, C797S)) Monitoring of response
NGS						
Whole genome sequencing	Sequencing of the full genome		NA	Discovery of new targets (fusion genes involving intronic areas)	Risk of false positives (poor specificity) Risk of identifying germline mutations	None in routine, exploration of new targets in research (fusion genes involving intronic areas)
Whole exome sequencing	Sequencing of the full exome (coding regions)			Discovery of new targets or mechanisms of resistance	Heavy bioinformatics Low sensitivity	Tumour mutation burden (but usually replaced by large gene panels)
Panels (hybrid capture) Panels (amplicon sequencing)	Capture and hybridisation to probes of pre-determined regions of interest, then sequencing PCR amplifications of hotspots/exons/genes of interest, then sequencing	Depend on the gene panels (usually hotspots, exons or full genes in 30–400 genes)	Sensitivity 70–90% for SNVs Sensitivity 50–80% for fusions Specificity 65% for hybrid capture Specificity >99% for amplicon	Interrogates simultaneously pre-determined genes of interest Comprehensive detection of known and unknown mutations Detection of SNVs, CNVs, fusions Lower cost and less bioinformatics data compared to whole genome sequencing or whole exome sequencing	Imperfect specificity and concordance with tissue, in particular for low AF variants PCR amplification can bias CNVs and AFs Unable to detect fusions without prior knowledge of partners	Initial and resistance genotyping (focusing on genes of therapeutic interest) Tumour mutation burden (large, >300 genes panels)

ddPCR: digital-droplet PCR; NGS: next-generation sequencing; NA: not available; SNV: single-nucleotide variation; CNV: copy-number variation; AF: allelic fraction.

Data analysis

Sequencing will generate a massive amount of raw genomic data. Each read generated by the sequencing is assigned to a genetic location and aligned to the reference genome to detect any alteration [37]. In the field of liquid biopsy, variant calling is very challenging, and low AFs (<0.5%) of true mutations are particularly difficult to discern from sequencing artefacts [32]. Fusion genes will generate split reads, where both ends are mappable, but not the in-between (and potentially wide) sequence making them difficult to identify. Moreover, while hybrid capture has the ability to detect fusion without prior knowledge of the gene partner, amplicon-based NGS only covers previously known rearrangements.

How to interpret plasma NGS data

False negatives are the first limitation of cfDNA genotyping. If technical limits of the genotyping platforms can be a potential barrier, the main limitation remains the lack of DNA shed, which is strongly linked to the number and sites of metastases [24, 38].

The interpretation of "false positives", assuming tumour tissue as a reference, can be more challenging, since they are not necessarily due to sequencing errors. As previously reported, a "false positive" can be the consequence of tissue heterogeneity, the molecular profile of a tissue biopsy potentially missing a clone present in other tumour sites (figure 1). Tumour temporal and spatial heterogeneity will become increasingly complex through the different lines of treatments, by two mechanisms potentially coexisting: selection of a minor clone (*i.e.* T790M mutation) and acquisition of *de novo* resistance mutations [36, 39, 40]. Concordance between tissue and plasma will thus become lower, and the physician will interpret the result of the assay depending on the relevance of the mutation within the context of the cancer (the example of T790M is detailed in the next section).

But a false positive can also be due to the detection of a variant linked to another tumour, or to pre-cancerous conditions. DNA shed from clonal haematopoiesis is the most common situation generating cancer-associated genes mutations (*TP53*, *KRAS*, *JAK2*) [41, 42]. Paired genotyping of peripheral blood cells can discriminate clonal haematopoiesis from cancer-derived variants.

Current and future applications of cfDNA for NSCLC genotyping

cfDNA to guide and monitor genotype-directed therapies

For the initial genotyping of the tumour, tissue is still the gold standard for NSCLC genotyping. However, technically challenging anatomy, biopsy complications, insufficient tumour tissue, low turnaround time, and sampling errors due to tumour heterogeneity limit this approach. Several plasma assays have been approved for *EGFR* when the genotyping cannot be assessed on these samples. Their specificity is strong enough to allow the prescription of EGFR-TKIs based on this result. However, the sensitivity is imperfect (∼70%) [43] and a negative result must be confirmed by a tissue biopsy, which will enable wider genotyping, including gene rearrangements (*e.g. ALK*, *ROS1*, *etc.*). To date, only *EGFR* mutational status is routinely assessed by a blood-based test.

Physicians are increasingly facing situations where there is a lack of tissue, given the paradox between an increasing number of targetable genotypes to track and the parallel development of minimally invasive bronchoscopic tools (EBUS-TBNA, radial EBUS-guided biopsy, electromagnetic navigation) that give access to small specimens [44–46]. Plasma NGS can noninvasively rapidly interrogate the genomic landscape of the tumour and, as previously proposed, targeted panels including genes of interest are preferred.

At progression, liquid biopsy constitutes a very appealing option to track mechanisms of resistance [27, 34]. This setting is different to initial diagnosis because tumour biology is more heterogeneous and subclonal [47]. The analysis of specificity is very tricky in this setting. In cases of *EGFR* mutated tumours, Oxnard *et al.* [27] showed that 31% of patients who test negative for the presence of T790M in tissue are positive in plasma (−/+). However, albeit lower than tissue/plasma (+/+) or tissue/plasma (+/−), these patients benefit from osimertinib treatment, establishing that these apparent false positives are not sequencing errors but the result of tumour heterogeneity [27]. The modest outcomes of patients designated as −/+ suggest more heterogeneous resistance, with the presence of other competing, coexistent clones. The relative AF of T790M (T790M AF/sensitising mutation AF) can be an additional tool in interpreting a plasma test, that informs the clinician whether T790M is the dominant mechanism of resistance or a subclonal phenomenon within a heterogeneous biology (T790M relative AF <10%). Also, the detection of the driver at high AF confirms that ctDNA is shed by the tumour and makes a negative T790M result more likely than a true negative, even if its presence as a minor subclone is still possible. Conversely, a test negative for both driver and T790M is not informative because it could potentially be linked to the absence of DNA shed, and indicates the need for tissue biopsy. A negative result must also be validated by tissue sampling, which will enable the detection of other mechanisms of resistance, not

covered by currently approved cfDNA assays (small cell carcinoma transformation, *MET* or *HER2* amplification, *PIK3CA* or *BRAF* mutations).

Overall, ∼30% of T790M-positive tissues are missed by plasma due to a lack of sensitivity/DNA shed, and ∼30% of cfDNA T790M-positive results would test negative on tissues due to tumour heterogeneity, making these two approaches complementary [27].

Oxnard *et al.* [27] proposed a new paradigm for detecting T790M mutations at progression: plasma genotyping should be proposed as a first-line screening test, with a tumour biopsy needed in the case of a negative result. Targeted plasma NGS covering a wider range of potential mechanisms of resistance to EGFR-TKIs (*EGFR*, *MET*, *BRAF*, *PIK3CA*, *HER2*) could, however, prevent the need for this repeat biopsy in future, with the only resistance mechanism that cannot be found in blood-based assays so far being small-cell transformation.

Monitoring plasma response throughout osimertinib treatment using amplicon-based NGS highlights the complexity of resistance biology in some cases where concomitant, competitive resistant clone (*KRAS*, *BRAF*, *PIK3CA*) can be seen in addition to T790M [36, 48]. This highlights the limitations of assays that only target T790M and can probably be extrapolated to any TKI resistance analysis, including front-line osimertinib, EGFR-TKIs targeting C797S, and other genotype-directed therapy [36]. The resistance to *ALK* inhibitors such as crizotinib is also too heterogeneous for targeted PCR-based assays, involving multiple on-target mechanisms (with variable sensitivities to second or third-generation anaplastic lymphoma kinase (ALK)-TKIs) also in addition to the upregulation of alternative pathways [49].

Analytical validation studies have now accumulated and clinical validation is strongly needed. Aggarwal *et al.* [50] demonstrated that plasma and tissue genotyping were complementary, with 20% of targetable variants detected in blood but not tissue, with sensitivity to the corresponding agents. Similarly, two recent prospective studies confirmed the complementary role of tissue and ctDNA for NSCLC genotyping [51, 52], with plasma having a lower turnaround time [52].

Potential future applications of cfDNA to guide and monitor immune therapy

While cfDNA has been widely studied in the field of targeted therapy, its potential to guide and follow the response to immune therapy is just beginning to be evaluated [53].

Tumour mutation burden (TMB) can be estimated from cfDNA NGS, with good concordance with tissue NGS provided that ctDNA is detected [54, 55]. In the only study showing discordant results between blood TMB and tissue TMB, two different platforms/panels were used for cfDNA and tissue [56]. A high blood TMB has proven to be correlated with response to inhibitors of programmed cell death (PD)1 and its ligand (PD-L1) [54, 57], in particular with NSCLC and atezolizumab in the POPLAR and OAK trials (high blood TMB being defined as >16 SNVs detected among 394 genes) [54, 58]. Of note, blood TMB is better correlated with metastatic tissue TMB (0.9 Pearson correlation *versus* 0.8 overall) than primary tumour, and concordance is better in cases with high ctDNA concentrations [59]. Also, in the study by Fabrizio *et al.* [54], the concordance was much better (64% positive agreement) when using a large gene panel (394 genes) than when using a smaller one (62 genes, 17% positive agreement).

However, the clinical adoption of TMB is challenging due to the high costs associated with whole-exome sequencing or broad panels. We performed targeted, amplicon-based, NGS of plasma cell-free DNA in 86 patients treated with PD1 inhibitors, using limited gene panels (n=36). Focusing on specific molecular determinants of response (*KRAS* and/or *TP53*) [60], or resistance (oncogenic drivers, *STK11*, *PTEN*) [60–63] to ICIs, we built a simple algorithm that could predict durable outcomes of patients with advanced NSCLC treated with immunotherapy [64].

Monitoring of response is another application of cfDNA during ICI treatment. Several reports have demonstrated a good correlation between ctDNA kinetics and clinical response. A complete or partial clearance at 4–8 weeks was strongly predictive of a durable response, whereas persistence of ctDNA had a detrimental impact [64–66]. We also showed that early changes in circulating tumour DNA burden has the ability to discriminate between pseudo-progression and true progression [67].

Other applications of cfDNA are likely to emerge in the near future, such as the detection of minimal residual disease (MRD) for adjuvant immunotherapy, or detection of mechanisms of resistance, like acquired JAK1/2 or B2M mutations [53].

Potential applications in early stage NSCLC: screening and residual disease

Screening is probably the most challenging area in lung cancer. The large National Lung Screening Trial [68] and more recently, the NELSON trial demonstrated that the use of computed tomography (CT) scans among asymptomatic men at a high risk for lung cancer led to a 26% reduction in lung cancer deaths at

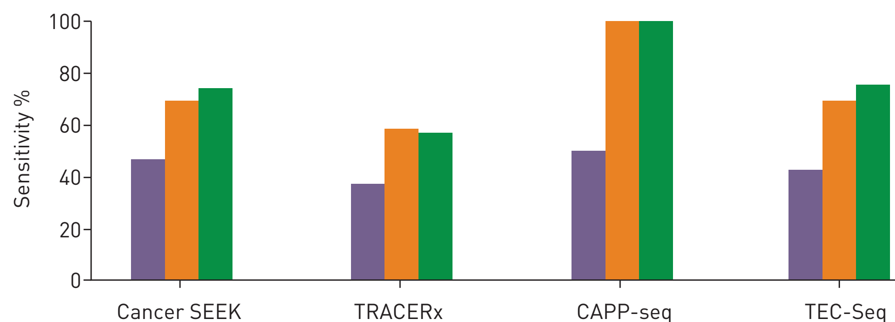
10 years of study follow-up (41% for women) [69]. Nevertheless, because lung nodules have many benign differentials, this examination generates a considerable number of false positives and leads to useless and dangerous (28% complications in the NSLT trial) invasive diagnostic biopsies or even surgeries. Combining this high sensitivity of CT scans with the specificity of liquid biopsy is very promising, but it is limited by the fact that the ctDNA shed is either absent or very low in the earlier stages.

A screening cfDNA assay should cover a sufficiently wide array of recurrent mutations to capture all NSCLC tumours, but should stay focused on targeted areas that are of great concern and need to be banished in the screening setting.

The principles and sensitivity of the four main platforms investigated for this purpose so far are reported in figure 2. Even the most sensitive assay, such as CAPP-seq, only detects 50% of stage I diseases (100% sensitivity for stage II and III) [70]. The CancerSEEK assay, combining genomic analysis of 16 genes in ctDNA and eight protein biomarkers, demonstrated compelling results in detecting eight types of nonmetastatic cancers (lung, colorectal, ovary, breast, liver, pancreas, oesophagus and stomach) [71]. However, while the test could reliably detect 70% of stage I–III cancers, its sensitivity for stage I cancers was low (43%), with lung and oesophagus showing the lowest rates of detection. Also, because lung cancer does not have a specific circulating protein marker, determining the site was only possible in 39% of cases *versus* 84% for colorectal, 79% for ovary and 81% for pancreatic cancers. Finally, the remarkable specificity (false positive rate <1%) may be higher in a population of healthy people (*i.e.* in a real screening setting).

One other limitation of early detection of cancer is the possibility to detect mutations in cfDNA that are not derived from tumour but clonal haematopoiesis, a false positive result that can be avoided by combining peripheral blood cells and cfDNA genotyping [41].

MRD is a term extrapolated from haematological disorders, which refers to the detection of any tumour-derived material in the blood after curative treatment. CAPP-seq has been shown to be highly effective for MRD detection after treatment of localised lung cancer (94% of patients with recurrence had ctDNA detected in blood). However, most of them had stage II–III disease (33 out of 40) [72]. Again, it is more challenging here for stage I diseases because of low/no DNA shed. However, unlike in the screening setting, it is possible to access the molecular profile from the initial tumour, and thus follow the patient with simplified and personalised, multiplex PCR assays. One of the best examples is in breast cancer. Using the mutations detected in the tissue after surgery, a patient-specific digital PCR assay is designed to track MRD through time. The detection of ctDNA with this methodology can predict metastatic relapse with high accuracy (HR 25.1) [73]. Using a similar approach, the detection of MRD after stage II colon cancer curative surgery was also found to be a strong predictor of relapse [74].



	Cancer SEEK#	TRACERx¶	CAPP-seq+	TEC-Seq§
Stage I	43	37	50	45
Stage II	69	59	100	72
Stage III	74	57	100	75

FIGURE 2 Summary of the technical properties and performances of the four main plasma genotyping platforms studied for early stage nonsmall cell lung cancer detection. #: early stages: hybrid capture, plasma next-generation sequencing (NGS) (16 genes) and 8 protein markers. ¶: minimal residual disease (MRD): plasma NGS, patient-specific multiplex PCR (10 to 22 single-nucleotide variations), subclonal evolution. +: early stage MRD: hybrid capture, plasma NGS (139 genes). §: early stage MRD: hybrid capture, plasma NGS (58 genes).

Given the low benefit of adjuvant chemotherapy in NSCLC (5% absolute benefit [75]), new approaches to detect clinically indiscernible MRD and guide adjuvant therapy are strongly needed. The TRACERx team analysed cfDNA data from the first 100 patients enrolled in their study from diagnosis through treatment (including curative surgery) and beyond [76–78]. Personalised sequencing assays were built and used for each patient from multi-region exome sequencing of the tumour. Although potentially costly and time consuming, these assays had remarkable sensitivity and specificity (as the team set a threshold of two or more variants for positivity). While some predictors of DNA shed were already established, which included tumour size, number of metastatic sites, stage and organ of origin [24, 28, 79], this study discovered that necrosis, lymphovascular invasion, Ki67 labelling index, tumour size and nonadenocarcinoma status all predict ctDNA shed. However, they only identified tumour cfDNA in 48% of patients with early stage NSCLC, and discussed a theoretical limit of DNA shed for tumours of a given size, estimating that tumours <10 cm^3 will not have detectable ctDNA [77]. This work confirms that plasma genotyping can detect MRD/recurrence weeks to months before imaging (median 70 days). Finally, in many patients this assay identified the phylogenetic subclone that was responsible for recurrence.

Methylation is an early and frequent epigenetic alteration that can be detected in cfDNA, including in plasma. A methylation panel of six genes showed a sensitivity of 72% (31 out of 41) for the detection of stage Ia NSCLC, but with a poor specificity (12 out of 42 control patients tested positive) [80]. A prospective trial is ongoing to investigate the ability of ctDNA mutations and methylation monitoring to detect MRD after surgery for stage Ia–III NSCLC [81].

In addition to the inherent low DNA shed, another limitation to the integration of plasma genotyping in early stage disease is the lack of CT targets to monitor treatment efficacy. If MRD detection was to guide systemic therapy, exclusive plasma monitoring should be chosen, as in chronic myeloid leukaemia.

Circulating tumour cells

Pathophysiology

The release of CTCs into the bloodstream benefits from a change in the tumour cell phenotype, characterised by a loss of the epithelial markers (cytokeratin filaments and E-cadherin) and the gain of some mesenchymal markers such as vimentin. This epithelial–mesenchymal transition (EMT) increases the plasticity of CTCs, facilitating migration and invasion [82, 83]. CTCs may migrate as clusters of cells, called circulating tumour microemboli, which are likely to survive better in the bloodstream than isolated CTCs [84]. They can also bring their own "soil" (stroma cells from the primary tumour) to increase their metastatic potential [85, 86].

Isolation/enrichment and analysis

Two main approaches have been developed for CTC isolation: 1) methods based on antigen expression (antigens not expressed on blood cells, but not specific to tumour cells); and 2) methods based on biophysical characteristics (density and size, higher in CTCs than in blood cells, along with cytopathological criteria of malignancy) [77, 82–86].

Cellsearch is a widely used and US FDA-approved method that isolates cells from blood using epithelial specific antibodies (epithelial cell adhesion molecue-positive selection, CD45-negative selection) [87]. However, malignant CTCs frequently lose their epithelial antigens and acquire some mesenchymal markers during EMT [88], and not all circulating epithelial cells are malignant, explaining the lack of sensitivity and specificity of these approaches [89, 90].

Isolation by size of epithelial tumour cells (ISET; Rarecells, Paris, France) is based on the isolation of CTCs according to their size (superior to 20 μm microns compared to 8–10 μm for white blood cells) [91, 92] and enables a reliable cytomorphological analysis of the isolated cells [91] and immunocytochemical, immunofluorescence or *in situ* hybridisation to detect rearrangements or amplifications [93, 94].

The microfluidic technologies probably represent the most appealing approach for CTC isolation and have been reported since 2007 with the "CTC-ship" [95]: CTCs are captured in antibody (epithelial cell adhesion molecule)-coated microposts under precisely controlled laminar flow conditions. This allowed for the detection of CTCs in 99% (115 out of 116) of patients with various solid tumours [95]. Pasortix uses a similar approach but isolates tumour cells based on compressibility as well as their size (microfluidic technology). This technology has been used in patients with prostate and breast cancer for single-cell genotyping, revealing molecular heterogeneity across CTCs [96].

The Vortex technology also exploits microfluidics and uses laminar microscale vortices to isolate and concentrate CTCs from blood, based on their physical properties (size, shape and compressibility) [97, 98]. This approach has the advantages of being automated, being independent of antibody expression (thus

avoiding false negatives due to EMT), and results in high purity CTC samples with low white blood cell contamination and preserved cells, facilitating further molecular analyses or culture [97, 98].

Endeavours to improve the sensitivity of these technologies and increase the sample purity of CTCs, but also to miniaturise the microfluidics platforms are ongoing [99].

Current and potential applications of CTCs for NSCLC

Screening

In early stage cancer, the CTC detection rate is usually low, <30% [100, 101]. In the case of Cellsearch enrichment, this low sensitivity, combined with a poor specificity (any inflammatory disease can release circulating epithelial cells), probably makes this approach unsuitable for cancer screening. ISET has a better specificity and is currently being evaluated by Ilie *et al.* [102] as a screening tool in high-risk populations. In a cohort of 168 patients with COPD, CTCs were detected using ISET (RareCells, Paris, France) in five patients who were subsequently followed up with a yearly CT scan. All of these patients developed a lung nodule 1–4 years after, corresponding to stage Ia NSCLC that could be treated surgically, while none of the other patients with COPD or control patients developed lung cancer. Based on these positive results, the large AIR trial was launched. This prospective, multicentric study (21 centres) has enrolled 600 patients with COPD. Yearly screening with both low-dose CT scans and ISET blood treatment has been performed [103]. Results are not yet published. CTC detection after radical treatment (MRD) to guide adjuvant therapy or early detection of relapse is another potential application [84].

Prognosis

While the most robust data have been reported in patients with breast [104] and colorectal cancer using Cellsearch [105, 106], several studies have also established the burden of CTCs as an independent prognostic factor in NSCLC [107]. In particular, a high CTC count before, during or after surgery correlates with a worse prognosis, and could influence the adjuvant therapy decision [108–111].

Monitoring of response

A large study was performed on 326 patients with metastases enrolled in phase I studies in order to evaluate the potential of early CTC changes (Cellsearch) to predict response to treatment. However, CTC kinetics did not correlate with response according to RECIST criteria. Sensitivity and specificity of CTC count variations for response prediction was only 41% and 80%, respectively [112]. Accurate quantitative follow-up may be difficult due to the very low number of CTCs that are usually detected, and given the great potential of cfDNA to monitor tumour burden, CTCs should not be used for response follow-up.

Genomics

The detection of *EGFR* mutations in DNA extracted from CTCs [113] and the complementary role of cfDNA and CTCs for resistance genotyping, as demonstrated in several studies (in acquired resistance to EGFR-TKI in particular), created a strong interest in CTCs for genomics [79, 114] which diminished with the accumulation of highly sensitive, specific and rapid cfDNA genotyping platforms. CTC genotyping is less sensitive and more laborious [25, 26] and thus probably less suited for translation into routine. Although it is now widely accepted that cfDNA should be favoured over CTCs for detecting point mutations, CTCs may still play a role in detecting chromosomal rearrangements or CNVs because they can be subjected to immunocytochemical and fluorescence *in situ* hybridisation analyses, as demonstrated for *ALK* rearrangements and *MET* amplifications [115, 116]. Nevertheless, the ability of plasma NGS to detect a wide range of genomic alterations, including SNVs, CNVs and chromosomal rearrangements with high sensitivity has now been demonstrated [30–32, 36, 50, 117]. The yield, in addition to the cost and turnaround time of these two approaches, should be compared. Single-cell analysis of CTCs to better decipher resistance [118] is sometimes reported as an advantage of CTCs over ctDNA [119]. In particular, this approach has demonstrated that both on-target and off-target resistance mechanisms can be identified in the same CTC after acquired resistance to ALK–TKI [120]. It has, however, limited clinical impact and is technically highly challenging.

PD-L1 expression

Another potential application of CTCs is PD-L1 analysis, which cannot be explored in cfDNA. The hypothesis that PD-L1 expression on CTCs could be used as predictive marker of response to ICIs has unfortunately not been confirmed to date. Studies on PD-L1 expression on CTCs in advanced NSCLC have accumulated and while the feasibility is now well established, the rate of PD-L1^{+} CTCs has always been found to be very high and associated with poor prognosis, without a clear scientific explanation [121–128]. Only Ilie *et al.* [94] were able to show good concordance between tissue and CTCs, but the rate of positive samples was surprisingly low and correlation with response to PD1/PD-L1 inhibitors could not be investigated. The relationship between CTCs and circulating immune cells (liquid microenvironment) is

extremely complex [129], making CTCs an interesting tool to study metastasis and immune escape mechanisms, but they seem far from being adopted into routine practice as a biomarker for cancer immunotherapy.

Drug screening

CTCs can be used *ex vivo* (CTC culture) or *in vivo* (CTC-derived xenograft) as an alternative to tissue patient-derived xenograft for drug screening. CTC-derived xenograft models have been studied by Hodgkinson *et al.* [130], mainly in small-cell lung cancer and will not be extensively reviewed here, particularly as they are much more challenging to generate from NSCLC-derived CTCs [131]. CTC-derived xenografts mirror the corresponding patient's response to chemotherapy [130], but not to immunotherapy (because the mice are deeply immunosuppressed).

Discussion

Is tissue still the issue?

In our opinion, liquid biopsy will never fully replace tissue biopsy, which will remain the gold standard for the pathological diagnosis of lung cancer, by clarifying the subtype of cancer using cytomorphological criteria and immunohistochemistry assays (adenocarcinoma *versus* squamous *versus* other). In addition, tissue sampling is sometimes the only way to assess the exact stage of cancer (*e.g.* EBUS-TBNA, mediastinoscopy, *etc.*) or to confirm disease recurrence. When some material is left after all the diagnostic steps, genotyping of DNA extracted from tissue is still the standard. A negative plasma test will always need tissue confirmation, due to its lack of sensitivity (inherent of the assay and due to the inconsistent DNA shed). Furthermore, in this context of immune therapy revolution, tumour biopsy will have more and more value, in both assessing PD-L1 expression on tumour cells and exploring the tumour microenvironment. Genetic mechanisms of primary or acquired resistance to ICIs have been reported, such as *JAK* and *B2M* acquired mutations, and may be detected in blood [132, 133]. However, acquired resistance to ICIs still needs to be explored and should involve dynamic changes in the microenvironment, such as loss of surface expression of major histocompatibility complex class I, changes in polarisation of immune cells, or involvement of other immune checkpoints, which are impossible to detect from a simple blood draw.

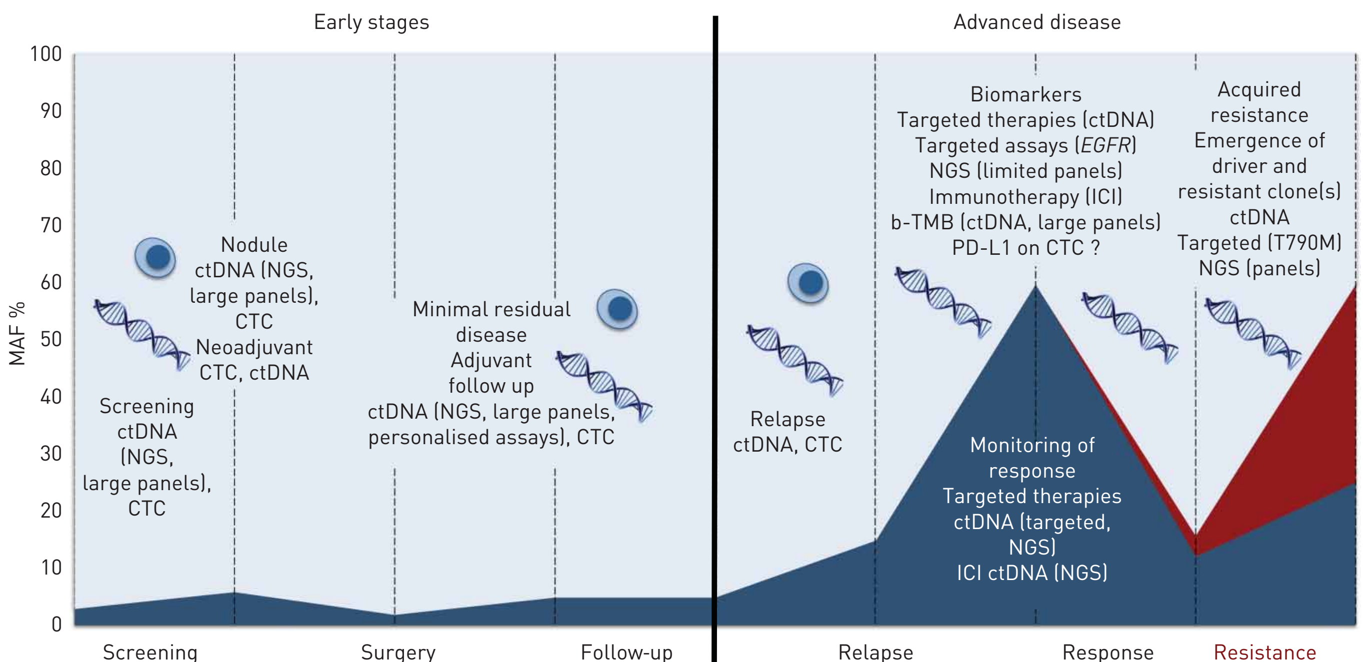


FIGURE 3 Potential applications of liquid biopsy in nonsmall cell lung cancer throughout treatment. In early stage disease, screening requires plasma next-generation sequencing (NGS) using large panels, with both high sensitivity (limited by low tumour shed) and perfect specificity, or highly specific circulating tumour cell (CTC) detection platforms. To discriminate benign from malignant nodules, plasma NGS could be useful and avoid invasive biopsies. Circulating tumour (ct)DNA or CTCs burden before surgery have the potential to help guide neoadjuvant therapy, while minimal residual disease detection after surgery (large or patient-specific NGS panels) may guide adjuvant therapy. In advanced stage disease, plasma genotyping is well established in the epidermal growth factor receptor (*EGFR*) setting, and NGS platforms allow for a wider genotyping at both diagnosis (including other oncogenic drivers detection and blood tumour mutation burden (b-TMB) estimation) and resistance. MAF: mutant allele frequency; ICI: immune checkpoint inhibitor; PD-L1: programmed-death ligand 1.

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Can liquid biopsy replace imaging for response monitoring?

Based on the observations that cfDNA kinetics accurately monitor tumour burden through treatment, it has been suggested that repeated liquid biopsy could outclass a CT scan follow-up. Different situations should be discussed. For example, if a patient does not show any clinical changes and their plasma does not show any detectable tumour DNA, perhaps clinical and blood monitoring alone can indeed replace imaging. However, if a patient reports new symptoms, regardless of whether their plasma shows an increase in ctDNA or not, a CT scan should be performed, because plasma could potentially miss a recurrence due to poor sensitivity.

Nevertheless, the most challenging and still unanswered situation is the case of a clinically stable patient showing re-emergence of ctDNA (recurrence of the driver and/or emergence of a resistance clone and/or detection of nondriver variants). In this case, a CT scan should be performed. If the scan confirms disease progression, treatment should obviously be adapted. However, it is still not known whether isolated plasma progression should lead to treatment modification. Some dedicated trials such as the APPLE-EORTC trial are investigating the particular case of T790M-driven acquired resistance, and may be extrapolated to other similar contexts [134]. Our feeling is that plasma progression predicts clinical progression associated with deterioration in both quality of life and performance status, which can compromise the initiation of further treatment. This suggests that treatment modification could be considered from the time when ctDNA emerges. The management of this situation will obviously depend on the "targetability" of the alterations detected in blood.

In conclusion, liquid biopsy has the potential to help manage NSCLC throughout all stages of lung cancer [135]: screening, MRD detection to guide adjuvant treatment, early detection of relapse, systemic treatment initiation and monitoring of response (targeted or immune therapy), and resistance genotyping (figure 3).

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