Journal of
Molecular Pathology

MDPI

Review

Liquid Biopsy for Biomarker Testing in Non-Small Cell Lung Cancer: A European Perspective

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Abstract: The development of targeted therapies has improved survival rates for patients with advanced non-small cell lung cancer (NSCLC). However, tissue biopsy is unfeasible or inadequate in many patients, limiting biomarker testing and access to targeted therapies. The increasing numbers of established and emerging biomarkers with available targeted treatments highlights the challenges associated with sequential single-gene testing and limited tissue availability. Multiplex next-generation sequencing (NGS) offers an attractive alternative and represents a logical next step, and in cases where the tumour is inaccessible, tissue biopsy yields insufficient tumour content, or when the patient's performance status does not allow a tissue biopsy, liquid biopsy can provide valuable material for molecular diagnosis. Here, we explore the role of liquid biopsy (i.e., circulating cell-free DNA analysis) in Europe. Liquid biopsies could be used as a complementary approach to increase rates of molecular diagnosis, with the ultimate aim of improving patient access to appropriate targeted therapies. Expert opinion is also provided on potential future applications of liquid biopsy in NSCLC, including for cancer prevention, detection of early stage and minimum residual disease, monitoring of response to therapy, selection of patients for immunotherapy, and monitoring of tumour evolution to enable optimal adaptation/combination of drug therapies.

Keywords: cfDNA; ctDNA; liquid biopsy; next-generation sequencing; non-small cell lung cancer; molecular diagnostics

Citation: Malapelle, U.; Tiseo, M.; Vivancos, A.; Kapp, J.; Serrano, M.J.; Tiemann, M. Liquid Biopsy for Biomarker Testing in Non-Small Cell Lung Cancer: A European Perspective. *J. Mol. Pathol.* **2021**, *2*, 255–273. https://doi.org/10.3390/jmp2030022

Academic Editor: Paul A. VanderLaan

Received: 29 June 2021
Accepted: 9 August 2021
Published: 18 August 2021

1. Introduction

Non-small cell lung cancer (NSCLC) is an aggressive, genetically heterogenous disease and is the leading cause of cancer death in Europe [1–4]. Five-year survival rates are approximately 26% for patients with stage IIIB disease and below 14% in stage IIIC/IVA/IVB disease [5]. Patients with tumours that harbour specific oncogenic driver alterations have demonstrated improved outcomes with new targeted therapies [6,7]. Population-level mortality data show a substantial improvement in survival over time, corresponding to the approval of targeted therapies [6] (beginning with the European Medicines Agency approval of erlotinib for epidermal growth factor receptor (*EGFR*)-mutated NSCLC in 2005 [8]).

Contemporary molecular analyses in patients with NSCLC comprise both the detection of oncogenic driver mutations to determine eligibility for targeted therapies [2] as well as testing for programmed death receptor ligand 1 (PD-L1) to guide immune checkpoint inhibitor therapy. In this review, we focus on the former: biomarker testing for oncogenic driver mutations to guide targeted treatment.

Several oncogenic drivers are predictive biomarkers and serve as therapeutic targets for patients with advanced non-squamous NSCLC, including *EGFR*, anaplastic lymphoma kinase (*ALK*), ROS proto-oncogene 1 (*ROS1*), B-Raf proto-oncogene (*BRAF*), rearranged during transfection (*RET*), neurotrophic receptor tyrosine kinase (*NTRK*), and hepatocyte growth factor receptor (*MET*) exon 14 skipping mutations [9,10]. Notably, the first targeted therapy for patients with NSCLC and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) exon 2 G12C mutations (sotorasib) has also recently received approval from the U.S. Food and Drug Administration (FDA) [11], as has amivantamab for patients with *EGFR* exon 20 insertions [12]. Novel targeted therapies in clinical development against other actionable biomarkers include receptor tyrosine-protein kinase erbB-2 (*ERBB2/HER2*) and neuregulin (*NRG1*) [9].

Despite guidelines recommending biomarker testing for all patients with advanced NSCLC adenocarcinoma [2], biomarker testing rates are still suboptimal [7,13]. There are multiple barriers to biomarker testing in clinical practice [9]; however, one important limitation is tissue availability. Considering the numerous established and emerging actionable biomarkers, sequential single-gene testing can potentially consume considerable tissue and time. Massive parallel sequencing, also known as next-generation sequencing (NGS), enables simultaneous sequencing of multiple biomarkers and represents a logical evolution to enable current and future precision oncology in NSCLC [10,14–17]. NGS represents a more reliable and efficient approach to biomarker testing compared with sequential single-gene testing [14,16]. In cases in which the tumour is inaccessible or the patient's performance status does not allow invasive tissue biopsy, liquid biopsy can provide valuable material for molecular diagnosis. Furthermore, the application of liquid biopsy for resistance monitoring has been established in the context of *EGFR* T790M mutations (and first- and second-generation EGFR tyrosine kinase inhibitor (TKI) therapy) and is likely to expand in line with the evolving armamentarium of NSCLC-targeted therapeutics. This has been evident with the recent FDA approvals of sotorasib and amivantamab together with liquid biopsy companion diagnostics, such as Guardant360® CDx (Guardant Health, Redwood City, CA, USA; for both sotorasib and amivantamab) and Therascreen® (Qiagen, Hilden, Germany; amivantamab only) [11,12]. Similarly, liquid biopsy may prove useful for minimum residual disease (MRD) monitoring in solid tumours. Taken together, these factors demonstrate that liquid biopsy NGS may help to increase detection of actionable alterations and improve delivery of targeted therapies for patients with NSCLC and other solid tumours, and as such, it can be seen as complementary to tissue-based biomarker testing [18–20].

In the present study, we explored the role of liquid biopsy (i.e., the analysis of circulating free DNA (cfDNA)) as a complementary approach to increase rates of molecular diagnosis in NSCLC in Europe, with the aim of identifying the most appropriate targeted therapy for each individual patient.

2. Which Biomarker Tests Are Currently Recommended in Europe for NSCLC-Targeted Treatment?

European Society for Medical Oncology (ESMO) guidelines (2020) recommend that all patients with advanced adenocarcinoma of the lung should be tested for oncogenic drivers, including systematic testing of *EGFR* and *BRAF* mutations and analysis of *ALK*, *ROS1*, and *NTRK* rearrangements [2]. Similarly, National Comprehensive Cancer Network (NCCN) guidelines recommend testing of oncogenic driver mutations (*EGFR*, *KRAS*, *BRAF*, *MET* exon 14), rearrangements (*ALK*, *ROS1*, *NTRK1/2/3*, *RET*), and analysis of PD-L1 expression levels in patients with advanced or metastatic NSCLC [21]. There are some

regional variations in terms of testing guidelines; European NSCLC biomarkers guidelines were recently reviewed in detail [9].

While sequential, single-gene biomarker testing remains the standard in several European countries, recent pharmacoeconomic analyses conducted from a European perspective suggest that, in the long run, NGS may be less costly than sequential single-gene testing, with savings increasing according to the number of patients and genetic alterations tested [15,17]. ESMO guidelines recognise the crucial role of NGS in the molecular work-up of patients with NSCLC [2]; NGS is deemed appropriate for detection of *EGFR* and *BRAF* mutations (subject to validation and external quality assurance) and *NTRK* rearrangements (validated by immunohistochemistry) and is cited as an emerging technology for the detection of *ALK* and *ROS1* rearrangements [2]. Notably, the guidelines state that "If available, multiplex platforms (NGS) for molecular testing are preferable" [2]. Similarly, the ESMO Precision Medicine Working Group (PMWG) recommends that samples from a patient with advanced non-squamous NSCLC are profiled using NGS technology to detect ESMO Scale of Clinical Actionability (ESCAT) level I alterations (Table 1) [10].

Table 1. ESMO Precision Medicine Working Group recommendations for NGS in non-squamous NSCLC.

Gene	Alteration	Prevalence	ESCAT
Level I alterations: "The ESMO Precision Medicine Working Group recommends that a tumour (or plasma) sample from a patient with advanced non-squamous NSCLC is profiled using NGS technology in order to detect level I alterations"			
EGFR	Common mutations (*Del19*, *L858R*)		
	Acquired *T790M* exon 20	15% (50–60% in Asians)	IA
	Uncommon *EGFR* mutations (*G719X* in exon 18, *L861Q* in exon 21, *S768I* in exon 20)	60% of *EGFR* mutant NSCLC10%	IA IB
ALK	Fusions (mutations as mechanism of resistance)	5%	IA
MET	Mutations ex 14 skipping	3%	IB
$BRAF^{V600E}$	Mutations	2%	IB
ROS1	Fusions (mutations as mechanism of resistance)	1–2%	IB
NTRK	Fusions	0.23–3%	IC
RET	Fusions	1–2%	IC
Level II–III alterations: "There is no evidence that panels detecting genes with a lower level of evidence brings additional value from a public health perspective. They could be used only if the report ranks genomic alterations according to valid ranking systems (e.g., ESCAT, OncoKB) and on the basis of specific agreements with payers taking into account the overall cost of the strategy (including off label use of drugs) as compared with small panels."			
EGFR	Exon 20 insertions	2%	IIB
MET	Focal amplifications (acquired resistance on EGFR TKI in *EGFR*-mutant tumours)	3%	IIB
$KRAS^{G12C}$	Mutations	12%	IIB
ERBB2/HER2	Hotspot mutations Amplifications	2–5%	IIB
BRCA 1/2	Mutations	1.2%	IIIA
PIK3CA	Hotspot mutations	1.2–7%	IIIA
NRG1	Fusions	1.7%	IIIB

Adapted from [10], Ann Oncol, Vol. 31, Mosele, F., et al., Recommendations for the use of next-generation sequencing (NGS) for patients with metastatic cancers: a report from the ESMO Precision, pages 1491–1505, Copyright (2020), with permission from the European Society for Medical Oncology. Published by Elsevier Ltd. All rights reserved. Abbreviations: *ALK*, anaplastic lymphoma kinase; *BRAF*, B-Raf proto-oncogene; *EGFR*, epidermal growth factor receptor; *ERBB2/HER2*, receptor tyrosine-protein kinase erbB-2; ESCAT, European Society for Molecular Oncology Scale for Clinical Actionability of molecular Targets; ESMO, European Society for Medical Oncology; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *MET*, hepatocyte growth factor receptor; *NRG1*, neuregulin; *NTRK*, neurotrophic tyrosine kinase receptor; *RET*, rearranged during transfection; *ROS1*, ROS proto-oncogene 1; TKI, tyrosine kinase inhibitor.

3. How Many Patients Miss out on Biomarker Testing/Molecular Diagnosis in Europe?

Despite guideline recommendations, real-world data suggest that biomarker testing rates across Europe remain suboptimal for patients with NSCLC [7,13]. A recent analysis of data (2015–2019) from the German CRISP registry demonstrated that 12.4% of patients with advanced NSCLC were not tested for any biomarker, and overall testing rates in non-squamous NSCLC for *EGFR*, *ALK*, *ROS1*, and *BRAF* were 72.5%, 74.5%, 66.1%, and 53.0%, respectively [7]. In another analysis, 49%, 24%, and 44% of patients with newly diagnosed advanced (stage IIIB/IV) NSCLC did not receive any biomarker test in Italy, Spain, and Germany (2011–2016), respectively [22]. Assuming a conservative estimate of 80% of NSCLC patients (all stages) presenting with advanced-stage disease, annually, these percentages would translate to approximately 14,000, 4800, and 19,400 patients in Italy, Spain, and Germany, respectively. In the Spanish Lung Cancer Biomarker Testing Registry study, *ALK* was not tested in 20% of cases, and *ROS1* was not tested in over 40% of cases [23]. The situation is similar in the USA, as shown by a recent study by the MYLUNG Consortium™, which demonstrated that most patients (90%) received testing for at least one out of five biomarkers (*EGFR*, *ALK*, *ROS1*, *BRAF,* and PD-L1) prior to first-line therapy, whereas less than half had testing for all five [24]. In this study, the median time from diagnosis to first-line therapy was approximately five weeks, and the turnaround time from testing orders to results was approximately two weeks.

Results of the International Association for the Study of Lung Cancer (IASLC) global survey on molecular testing in lung cancer support these data on suboptimal biomarker testing rates [13]. Of the European respondents, 21% of medical oncologists and 43% of surgeons, pulmonologists, or radiologists, reported that under half of patients with lung cancer received molecular testing in their clinics or institutions.

Based on a conservative estimate of 10% of patients not receiving any biomarker testing, annually, approximately 40,000 patients with NSCLC in Europe do not have the chance to benefit from precision oncology.

4. Why Are Patients Missing out on Biomarker Testing/Molecular Diagnosis?

There are several barriers to biomarker testing in Europe, including access, reimbursement, and technical limitations; these have been reviewed in detail previously [9]. However, one key reason, which we explore in this article, is the challenge of obtaining sufficient tumour material via invasive tissue biopsy (Figure 1). Most patients with NSCLC present with advanced-stage disease and are not eligible for curative surgical treatment [25], contributing to a high prevalence of small-specimen biopsies. Furthermore, not all patients with NSCLC receive tissue biopsy. For example, in one study, 7% of patients with NSCLC in Italy and 15% of patients with NSCLC in Spain did not receive a tissue biopsy [22]. Consequently, the most frequently reported reasons for not conducting biomarker testing in NSCLC are insufficient amount or inadequate quality of tumour samples [7,13,22]. In the IASLC global survey on molecular testing in lung cancer [13], the main reasons for failure to obtain a molecular diagnosis were insufficient tumour cells provided (93% of respondents) and inadequate tissue quality (55%).

The acquisition of adequate tissue biopsies in NSCLC can be particularly challenging, as tumour sites in patients with advanced NSCLC are often difficult to access, and invasive biopsies are associated with risks, such as bleeding and pneumothorax [26,27]. For example, the incidence of pneumothorax in patients undergoing transthoracic needle biopsy is approximately 20% (ranging from 9–54%) [28]. Even when technically feasible, not all biopsies provide enough tissue for molecular diagnosis. Based on the few studies in the published literature [27,29–31], tissue biopsy may not be feasible in approximately 20% of patients, and when biopsy is feasible, samples are inadequate for testing (molecular diagnoses and/or histological diagnosis) in up to a quarter of cases; overall biopsy failure rates varied considerably, ranging from 8% to 43% (Table 2). The proportion of patients with initially unsuccessful tissue biopsies that were salvaged via re-biopsy were not reported

for European cohorts; however, studies conducted in Asia and North America suggest that the proportion of biopsies successfully salvaged varies from 30–75% [32–34].

Table 2. Rates of inadequate tissue biopsy in patients with advanced NSCLC (European cohorts).

Article	Summary	Biopsy Not Possible/ Not Carried Out (%)	Biopsy Sample Inadequate (%)	Overall Biopsy Failure Rate (%)
[22] (Italy, Spain, and Germany)	A chart review study including 515 patients in Italy, Spain, and Germany with advanced newly diagnosed NSCLC; 468/505 had a biopsy.	IB: 9%	NR	NR
[29] (France)	A prospective study of 2579 patients with advanced cancer (6% lung cancer) who progressed after 1 L treatment and were potentially eligible for molecular-based therapies. A total of 435 patients (17%) were withdrawn from the study (insufficient quantity or quality of tumour sample, n = 357; tumour sample < 10% tumour cells, n = 19; DNA extraction/quantity issues, n = 19).	NR	17%	17%
[30] (Greece)	A retrospective study of 72 patients with histologically confirmed advanced/metastatic NSCLC who received anti-EGFR TKIs (any line); most (56%) received 2L treatment. Five patients were excluded (7.8%) due to insufficient tumour in biopsies.	NR	7.8%	7.8%
[27] (France)	A retrospective study of 84 patients with lung cancer (93% adenocarcinoma) with documented *EGFR* mutation or *ALK* rearrangement who developed radiographic progression on TKIs. Thirty-nine patients (46%) underwent re-biopsy at the time of acquired resistance. Among the 39 re-biopsies, there was sufficient tissue for histopathological or cytological examination in 89.7% of cases; in three cases there was no tumour tissue, and one case showed necrotic tissue. Re-biopsy was considered feasible in 33 of 45 patients (73%) who did not undergo re-biopsy.	RB: 14%	RB: 10%	19%
[31] (France)	A prospective study of 100 patients with advanced NSCLC with RECIST-defined progression after 1 L therapy and a clinical indication for re-biopsy. Re-biopsy was not feasible in 18% of cases. Of the 82 patients who underwent re-biopsy, 94% could be analysed histologically. Upon histological examination, 18.3% of samples contained no tumour cells, and 7.3% of samples contained too few tumour cells for molecular analysis.	RB: 18%	RB: 26%	43%

Abbreviations: ALK, anaplastic lymphoma kinase; DNA, deoxyribonucleic acid; *EGFR*, epidermal growth factor receptor; IB, initial biopsy; L, line of treatment; NR, not reported; RB, re-biopsy; RECIST, Response Evaluation Criteria In Solid Tumours; TKI, tyrosine kinase inhibitor.

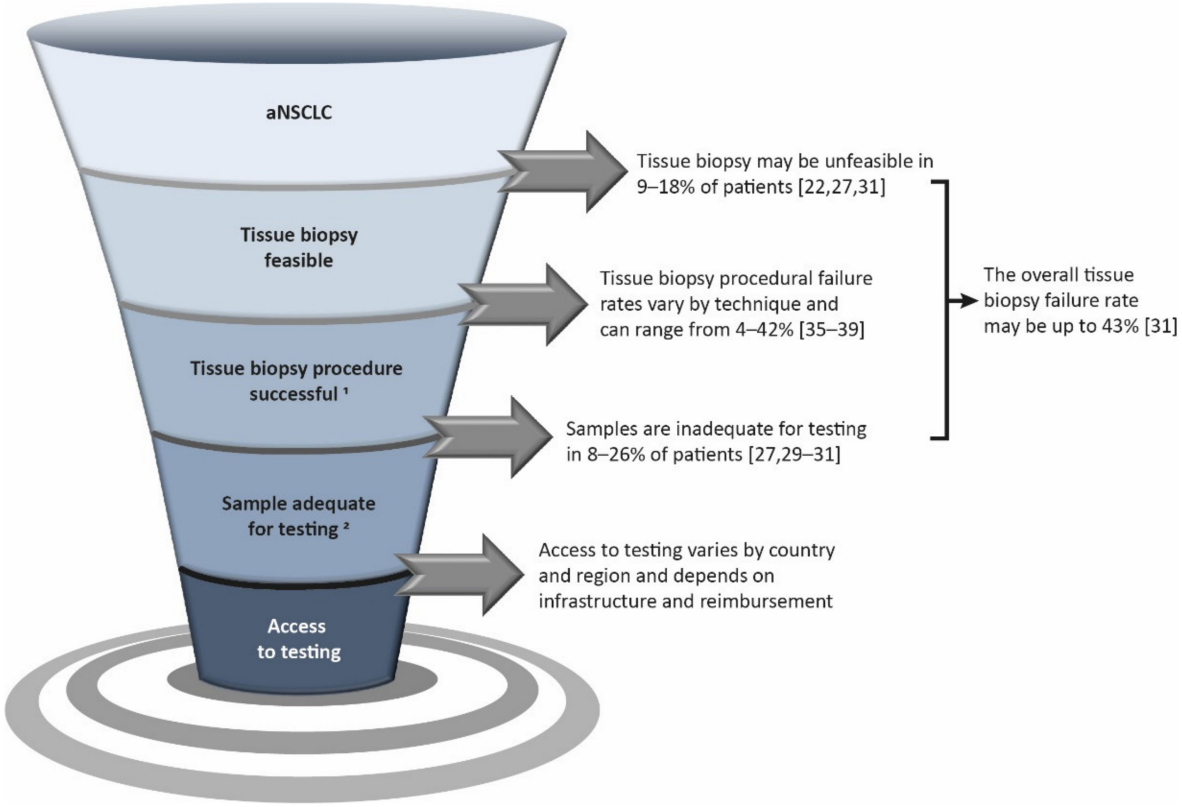

Figure 1. Reasons why patients miss out on biomarker testing/molecular diagnosis from tissue biopsy. Abbreviation: aNSCLC, advanced non-small cell lung cancer. [1] i.e., tissue sample successfully extracted from target lesion. [2] Molecular diagnosis and/or histological diagnosis [22,27,29–31,35–39].

Abbreviations: ALK, anaplastic lymphoma kinase; DNA, deoxyribonucleic acid; *EGFR*, epidermal growth factor receptor; IB, initial biopsy; L, line of treatment; NR, not reported; RB, re-biopsy; RECIST, Response Evaluation Criteria In Solid Tumours; TKI, tyrosine kinase inhibitor.

Diagnostic yields (i.e., the proportion of biopsies permitting a successful pathological diagnosis) can vary by biopsy technique and range from 58–70% with endobronchial ultrasonography (EBUS), 80–96% with CT-guided transthoracic needle aspiration, up to 94% with EBUS transbronchial needle aspiration (TBNA), and 93–94% with thoracoscopy [35–39]. The ongoing German, prospective, randomised PROFILER study examines the influence of biopsy technique on molecular genetic tumour characterisation in NSCLC [40]. As a guiding principle, the least invasive biopsy techniques that provide the maximum tissue yields should be used to establish molecular diagnoses and minimise the risk of bleeding and other complications.

The prevalence of tissue biopsy failure/inadequacy is estimated to affect up to 43% of patients with NSCLC, limiting optimal first-line treatment selection or subsequent treatments following disease progression. Liquid biopsy could serve as an integral solution for molecular diagnosis in patients with NSCLC with limited or inadequate tissue biopsies [41,42]. Considering our empirical estimate that 40,000 NSCLC patients do not receive biomarker testing annually—if we estimated that 40% of these were due to unfeasible/inadequate tissue biopsy (Table 2), that would equate to approximately 16,000 patients in Europe annually who could potentially benefit from liquid biopsy coupled with NGS to obtain a molecular diagnosis.

5. What Is Liquid Biopsy?

Liquid biopsy is the analysis of tumour-derived material from body fluids (e.g., blood, urine, saliva, cerebrospinal fluid, and pleural effusion) [41,43,44]. The most commonly tested analyte in blood is plasma-circulating cell-free DNA (cfDNA), which contains circulating tumour DNA (ctDNA; i.e., tumour-derived fragmented DNA in the bloodstream that is not associated with cells [45]). ctDNA makes up only a small fraction of cfDNA, but its levels can increase according to tumour burden and stage (I–IV) [46]. Therefore, assay sensitivity often increases as a function of tumour stage [46,47]. However, the relationship between tumour burden and ctDNA is not clear-cut, and ctDNA can be also impacted by tumour location, vasculature, necrosis, and cell turnover [48,49]. For example, patients with intracranial progression are less likely to shed tumour-derived DNA into the bloodstream compared with those with extracranial progression, due to the blood–brain barrier [48,50]. Although analysis of circulating mRNA can be carried out on liquid biopsy samples, this is significantly more challenging than ctDNA analysis. The clinical application of liquid biopsy-based mRNA detection of gene fusions or splice products is still limited, and further studies are warranted to assess the accuracy of RNA as a substrate for liquid biopsy.

The principal advantage of liquid biopsy is that it is minimally invasive and can be serially repeated to monitor disease evolution [48,51], including the development of resistance [2,52], the development of co-mutations [53], and for the more rapid detection of disease progression (i.e., ctDNA-defined progression) [54]. Liquid biopsies may be used for repeat biopsies at relapse [2], can reduce sampling error due to tumour heterogeneity [45], and are particularly useful when there is inadequate tumour tissue or when tumour tissue is inaccessible [52]. Compared with tissue biopsies, liquid biopsies are more convenient, less expensive (for single-gene tests), and the risk of procedural complications is lower due to the minimally invasive nature of sampling [45,55]. Additionally, turnaround times (defined as the length of time between test order date and report date) with liquid biopsy are approximately six days shorter than with tissue-based analysis [56]. Further to these advantages, similar clinical outcomes can be achieved with liquid biopsy-based molecular analyses guiding diagnosis and treatment as with standard tissue-based methods to guide treatment. Moreover, accuracy may be improved if both liquid- and tissue-based methods are used complementarily. For example, the addition of plasma NGS testing to routine management of advanced NSCLC increased detection of targetable alterations and improved delivery of targeted therapy [19]. Disease control rates for patients with NSCLC following liquid biopsy and amplicon-based NGS ctDNA analysis in first-line and in the relapse setting were similar to those observed with tissue biopsy [18]. Additionally, response rates to targeted treatment with tepotinib (following the detection of *MET* exon 14 skipping mutations) were comparable regardless of whether tissue biopsy or liquid biopsy underpinned the molecular work-up [57]. Finally, response rates following ctDNA-guided treatment in patients with advanced lung and gastric cancers were similar to those associated with tissue-guided targeted therapy [20].

Liquid biopsy is, however, associated with several limitations, which are summarised in Table 3 [41,42,58–64]. These include lower test sensitivity compared with tissue-based biomarker testing, the potential for misinterpretation of results (false positives) due to expansion of mutations in peripheral blood cells (clonal haematopoiesis), challenges in detecting certain types of gene fusion translocations or splice variant alterations, and the detection of germline variants. To help mitigate such risks, the application of liquid biopsy requires careful consideration, an appropriate methodology, and relevant controls [65].

Table 3. Limitations and considerations for liquid biopsy.

Limitations and Considerations	Details
Test sensitivity	• Liquid biopsy generally has lower test sensitivity versus tissue-based biomarker testing [41,42]. • Sensitivity is dependent on the method of analysis, genetic alteration of interest, and stage/type of disease (i.e., not all tumours shed sufficient amounts of DNA into the peripheral circulation for detection; e.g., 20% of stage IV patients do not shed ctDNA); the most sensitive assays provide approximately 85% sensitivity in advanced NSCLC [41,42].
Potential for false positives	• Clonal haematopoiesis (expansion of mutations in peripheral blood cells) can cause false positives if liquid biopsy results are misinterpreted [63,64]. However, it is relatively easy to control for this phenomenon [58].
Morphological transformation	• Morphological transformation (e.g., to small cell morphology) occurs in some patients (3–15%) [59] who acquire resistance to EGFR TKIs and requires tissue biopsy to be identified [60].
Challenges in detecting certain types of gene fusion translocations or splice variant alterations	• There can be higher false-negative rates for gene fusions with cfDNA NGS compared with tissue-based testing. This may be due to low ctDNA abundance and difficulties when intronic rearranged regions are involved in gene fusions [65]. • It can be difficult to define if the identified rearrangement involves a relevant gene fusion with the production of a chimeric protein with oncogenic activity. This may be assumed when two well-defined, recurrent partners of a gene fusion are involved in the rearrangement [65]. • NGS platforms permit simultaneous detection of mutations, indels, copy number variations, and genomic rearrangements. Specific advantages of NGS for ctDNA analysis include the ability to quantify gene copy number variations (e.g., gene amplifications) and to identify chromosomal rearrangements (e.g., oncogenic fusions) [41,42]. However, concerns around error rates with NGS are particularly relevant to the analysis of plasma ctDNA, where low variant allele frequencies can result in a single false-positive read impacting interpretation of the data; to some extent, this limitation can be addressed with the use of algorithms and error-proofing techniques [41,42]. • Liquid biopsy might detect indolent metastases, triggering treatment for a potentially inconsequential lung cancer, although this limitation also applies to low-dose computed tomography [61].
Detection of germline variants	• Liquid biopsy may detect germline variants [62]; however, it is relatively straightforward to control for their detection by parallel sequencing of non-tumour DNA. Although this approach is theoretically feasible, there are limitations: it is not possible to sort ctDNA from normal DNA, and very few centres procure normal tissue samples upfront or at the time of biopsy for parallel sequencing.

Abbreviations: cfDNA, circulating free DNA; ctDNA, circulating tumour DNA; DNA, deoxyribonucleic acid; EGFR, epidermal growth factor receptor; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor.

6. What Are the Current Recommendations for Liquid Biopsy in Europe?

Several European clinical guidelines/position papers recommend the use of liquid biopsy for patients with NSCLC in certain settings. While ESMO NSCLC guidelines state that liquid biopsy can be used for detecting *T790M* mutations in the context of EGFR TKI therapy [2], a report from the ESMO PMWG and the IASLC statement paper on liquid biopsy both provide a more general recommendation for the use of liquid biopsy in advanced NSCLC [10,41]. The ESMO PMWG report recommends that a tumour *or plasma* sample from a patient with advanced non-squamous NSCLC is profiled using NGS technology to detect ESCAT level I alterations (Table 1) [10]. Similarly, the IASLC statement paper recommends that frontline liquid biopsy testing may be considered in all patients who require molecular tumour profiling, particularly when tumour tissue is scarce, unavailable, or a significant delay is expected in obtaining tumour tissue [41]. Figure 2 summarises specific recommendations for treatment-naïve patients and patients who progress, either clinically or radiologically, during treatment with first- or second-generation EGFR TKIs [42]. In both populations, a positive finding provides sufficient

evidence to initiate appropriate targeted treatment; however, a negative liquid biopsy result should be considered inconclusive and requires secondary testing. The IASLC recommendations highlight the complementary nature of tissue and liquid biopsies in the NSCLC setting.

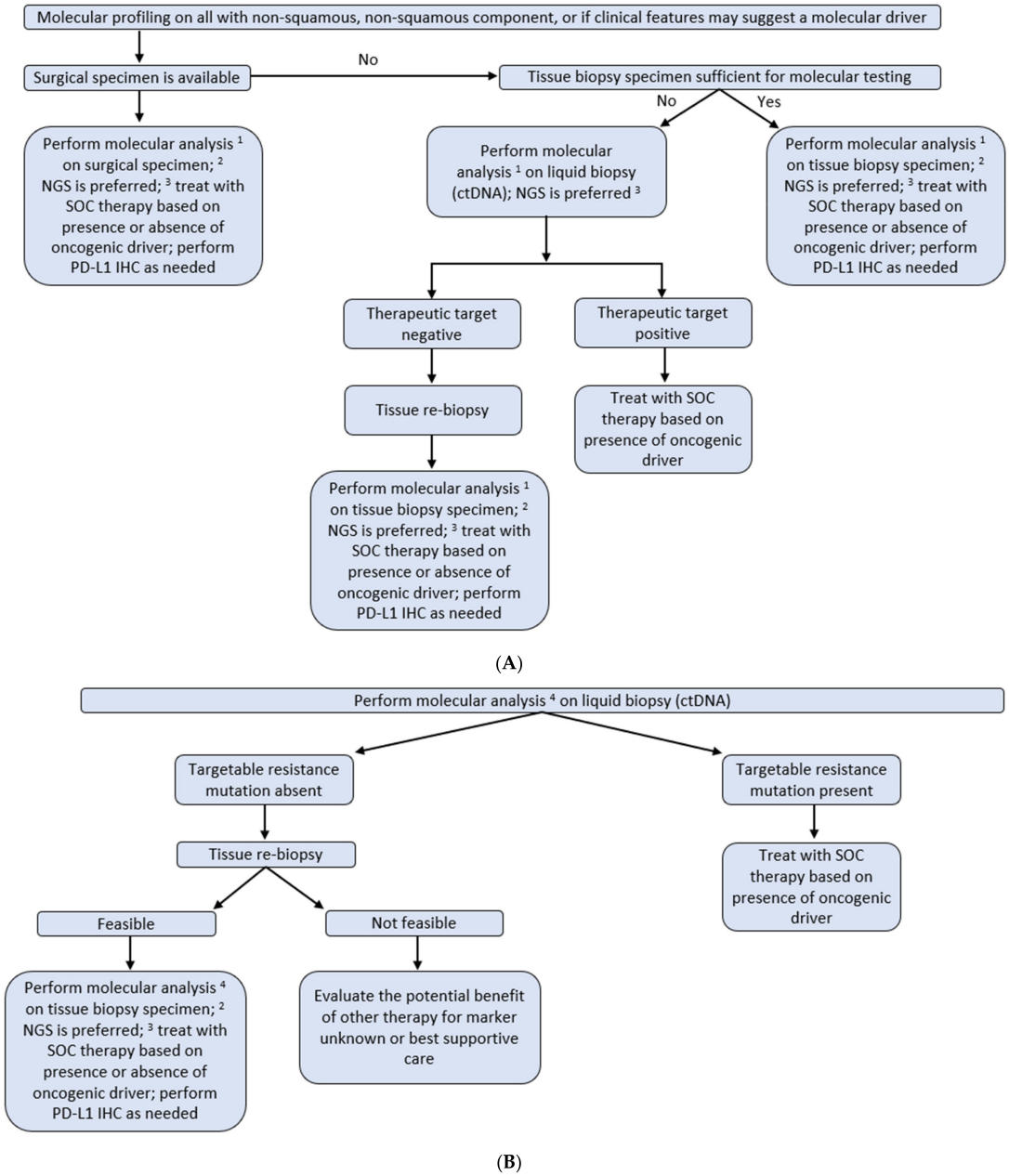

Figure 2. Liquid biopsy and tissue biopsy are complementary approaches for the molecular analysis of (**A**) patients with advanced, treatment-naïve NSCLC or (**B**) progressive or recurrent NSCLC during treatment with tyrosine kinase inhibitors. Reprinted from [42], J Thorac Oncol, Vol. 13, Rolfo, C., et al., Liquid biopsy for advanced non-small cell lung cancer (NSCLC): a statement paper from the IASLC, pages 1248–1268, Copyright (2018), with permission from the International Association for the Study of Lung Cancer. Published by Elsevier Inc. All rights reserved. Abbreviations: *ALK*, anaplastic lymphoma kinase; *BRAF*, B-Raf proto-oncogene; ctDNA, circulating tumour DNA; ddPCR, droplet digital polymerase chain reaction; *EGFR*, epidermal growth factor receptor; IHC, immunohistochemistry; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death ligand 1; *ROS1*, ROS proto-oncogene 1; SOC, standard of care. [1] *EGFR*, *ALK*, *ROS1*, and *BRAF* at minimum but a panel if available. [2] Strongly suggest tissue sparing to facilitate participation in clinical trials. [3] While NGS is preferred, based on availability, other validated assays are acceptable. [4] Cobas/ddPCR for *EGFR* mutation; NGS preferred for *ALK* and *ROS1*.

In contrast to European guideline recommendations for liquid biopsy NGS, single-gene liquid biopsies have been approved for several years in the USA, and pan-tumour, NGS-based liquid biopsy companion diagnostics (Guardant360® CDx and FoundationOne® Liquid CDx (Foundation Medicine, Cambridge, MA, USA)) were approved by the FDA in 2020 [66,67] (Table 4) [21,68,69]. Furthermore, according to updated molecular testing guidelines from the College of American Pathologists (CAP), IASLC, and the Association for Molecular Pathology (AMP), "the use of multiplexed genetic sequencing panels are preferred over multiple single-gene tests to identify other treatment options beyond EGFR, ALK, and ROS1" [68]. The liquid biopsy testing landscape is also rapidly changing in other countries; for example, in Japan, FoundationOne® Liquid CDx was approved in 2021 by the Ministry of Health, Labour and Welfare, and Guardant360® CDx has also been submitted for regulatory approval [70,71].

Table 4. Comparison between European and U.S. clinical guidelines on liquid biopsy for molecular diagnosis in NSCLC.

Guideline	Recommendations on Liquid Biopsy for Molecular Diagnosis	Recommendations on Multiplex Panel versus Single-Gene Testing (Recommendations Specific to Liquid Biopsy Are Bolded)
ESMO NSCLC [2]	• Liquid biopsy can be used as the initial test for detection of a T790M mutation. • If plasma testing is negative for T790M, tissue biopsy is strongly recommended to determine T790M status because of the risks of false-negative plasma results.	• If available, multiplex platforms (NGS) for molecular testing are preferable. • Multiplex, massively parallel, so-called NGS of various sorts is rapidly being adopted as the standard approach to screening adenocarcinomas for oncogenic targets (IIIA). • This multiplex approach is especially valuable and more efficient when the number of targets increases. • NGS techniques can be used (for blood monitoring); as more biomarkers are identified and validated, more NGS-based gene panels will become available.
ESMO PMWG [10]	• A tumour or plasma sample from a patient with advanced non-squamous NSCLC is profiled using NGS technology to detect level I alterations (see Table 1).	• It is recommended that a tumour (or plasma) sample from a patient with advanced non-squamous NSCLC is profiled using NGS technology in order to detect level I alterations. Considering the high frequency of fusions, RNA-based NGS or DNA-based NGS designed to capture such fusions are the preferred options. • Larger panels only recommended where specific agreements with payers consider the overall cost of the strategy, including drug cost.

NCCN [21]	• Plasma cell-free/circulating tumour DNA testing should not be used to diagnose NSCLC; tissue testing should be used to diagnose NSCLC.
	• The use of cfDNA/ctDNA can be considered in specific circumstances:
	  ○ If a patient is not medically fit for invasive tissue sampling.
	  ○ If there is insufficient tissue for molecular analysis and follow-up tissue-based analysis will be done if an oncogenic driver is not identified.
	• Recent data suggest that plasma cell-free/circulating tumour DNA testing can be used to identify *EGFR*, *ALK*, and other oncogenic biomarkers that would not otherwise be identified in patients with metastatic NSCLC.
	• Liquid biopsy may be considered at progression to detect whether patients have a T790M mutation; however, if plasma testing is negative, a tissue biopsy is recommended.
		• It is recommended at this time that, when feasible, testing be performed via a broad, panel-based approach, most typically NGS. For patients who, in broad panel testing, do not have identifiable driver oncogenes (especially in never-smokers), consider RNA-based NGS, if not already performed, to maximise detection of fusion events.

Table 4. *Cont.*

Guideline	Recommendations on Liquid Biopsy for Molecular Diagnosis	Recommendations on Multiplex Panel versus Single-Gene Testing (Recommendations Specific to Liquid Biopsy Are Bolded)
CAP/IASLC/AMP and ASCO [69]	• There is currently insufficient evidence to support the use of circulating cell-free plasma DNA molecular methods for the diagnosis of primary lung adenocarcinoma (no recommendation). • In some clinical settings in which tissue is limited and/or insufficient for molecular testing physicians may use a cfDNA assay to identify *EGFR* mutations (recommendation). • Physicians may use cfDNA methods to identify *EGFR* T790M mutations in lung adenocarcinoma patients with progression or secondary clinical resistance to EGFR-targeted tyrosine kinase inhibitors; testing of the tumour sample is recommended if the plasma result is negative (expert consensus opinion). • There is currently insufficient evidence to support the use of circulating tumour cell molecular analysis for the diagnosis of primary lung adenocarcinoma, the identification of *EGFR* or other mutations, or the identification of *EGFR* T790M mutations at the time of EGFR TKI resistance (no recommendation).	• The use of multiplexed genetic sequencing panels is preferred where available over multiple single-gene tests to identify other treatment options beyond *EGFR*, *ALK*, and *ROS1*.

Abbreviations: *ALK*, anaplastic lymphoma kinase; AMP, Association for Molecular Pathology; ASCO, American Society of Clinical Oncology; College of American Pathologists; cfDNA, circulating free DNA; ctDNA, circulating tumour DNA; DNA, deoxyribonucleic acid; *EGFR*, epidermal growth factor receptor; ESMO, European Society for Medical Oncology; NCCN, National Comprehensive Cancer Network; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PMWG, Precision Medicine Working Group; *ROS1*, ROS proto-oncogene 1; RNA, ribonucleic acid; TKI, tyrosine kinase inhibitor.

7. Beyond the Guidelines: What Does Liquid Biopsy and NGS Use in Europe Actually Look Like?

Liquid biopsies are not widely adopted or reimbursed in Europe [13]. Findings of the IASLC global survey on molecular testing in lung cancer show low uptake of liquid biopsy in Europe [8]. In the IASLC survey, while 90% of European respondents disclosed having requested liquid biopsies, and 80% stated that their laboratory offered testing on liquid biopsies, over two-thirds reported that $\leq$10% of molecular tests were performed on liquid biopsies in lung cancer [13]. Progress in implementing liquid biopsy into routine clinical practice in public hospitals depends on regional policy and reimbursement, and several innovative schemes are in place (e.g., in Andalucía, Spain) [72]. In Italy and the UK, liquid biopsy for *EGFR* mutation testing in NSCLC is generally reimbursed, but testing of other biomarkers is contingent on molecular tumour board (MTB) approval [73].

From a European perspective, liquid biopsy is currently viewed as complementary to tissue biopsy in the molecular work-up of NSCLC. Its use is typically limited to monitoring resistance to EGFR TKIs or when there is no documented tissue at baseline [2,18,19,57]. Targeted assays currently approved in Europe for liquid biopsies are limited to *EGFR* mutation testing and include the cobas *EGFR* Mutation Test v2 (Roche, Indianapolis, IN, USA) or Therascreen® assays [74].

NGS-based liquid biopsy brings additional access challenges in Europe. Currently, not all healthcare systems in Europe reimburse NGS, and uptake is not yet universal. Decisions around access and reimbursement for liquid biopsy NGS in Europe should consider both the analytical validity (i.e., ability of a test to identify a molecular target present in circulating DNA) and clinical utility (i.e., ability of a test to guide a therapeutic decision that determines an advantage in terms of overall survival and quality of life for the patient) [73]. In Italy, guidelines state that liquid biopsy can serve as an alternative approach for the identification of driver mutations in human neoplasms but should only be considered in the absence of available tumour tissue [73]. These guidelines emphasise that each NGS panel/method is associated with a unique sensitivity/specificity level. Therefore, each method must undergo appropriate validation, and the results obtained with one panel cannot be generalised [73]. Finally, it is recommended that the results of NGS tests

performed with large panels are discussed in a MTB to ensure correct interpretation [73]. A consensus statement on liquid biopsy from the Spanish Society of Pathology and the Spanish Society of Medical Oncology recognises the clinical validity of ctDNA testing for molecular profiling analysis and considers the advantages and limitations of NGS technology relative to quantitative polymerase chain reaction, droplet digital polymerase chain reaction (ddPCR), and BEAMing [75]. The statement also highlights the need to appoint an MTB to ensure optimal therapeutic decision making for each patient according to their genomic profile [75]. Several initiatives are in place in Europe that aim to increase patient access to liquid biopsy. For example, the NOWEL network in Germany (https://www.nowel.org/; accessed on 13 August 2021) offers comprehensive molecular diagnostics via liquid biopsy.

When access to NGS is limited, single-gene technologies like ddPCR can be used as a cost-effective approach to liquid biopsy; ddPCR can also serve as a confirmatory test alongside NGS [76]. ddPCR is accurate and sensitive with a limit of detection of 0.001% [47,77] and a limit of quantification of approximately 0.1% [78]. The sensitivity of ddPCR-based ctDNA detection varies according to mutation, ranging from 62–86% [47,79,80]. ddPCR-based liquid biopsy could bridge the gap for patients with insufficient tissue for biomarker testing in clinics without access to NGS, with the aim of reducing the proportion of patients with unknown molecular status. However, ultimately, NGS is expected to become the standard of care across Europe.

8. The Potential for Broader Adoption of Liquid Biopsy

Of our theoretical 16,000 patients annually in Europe who could benefit from molecular diagnosis using liquid biopsy (who might otherwise remain "biomarker status unknown"), given the epidemiologic frequency of actionable mutations [81–83], approximately 6560 otherwise unidentified patients annually could potentially benefit from current or investigational targeted treatments (Figure 3).

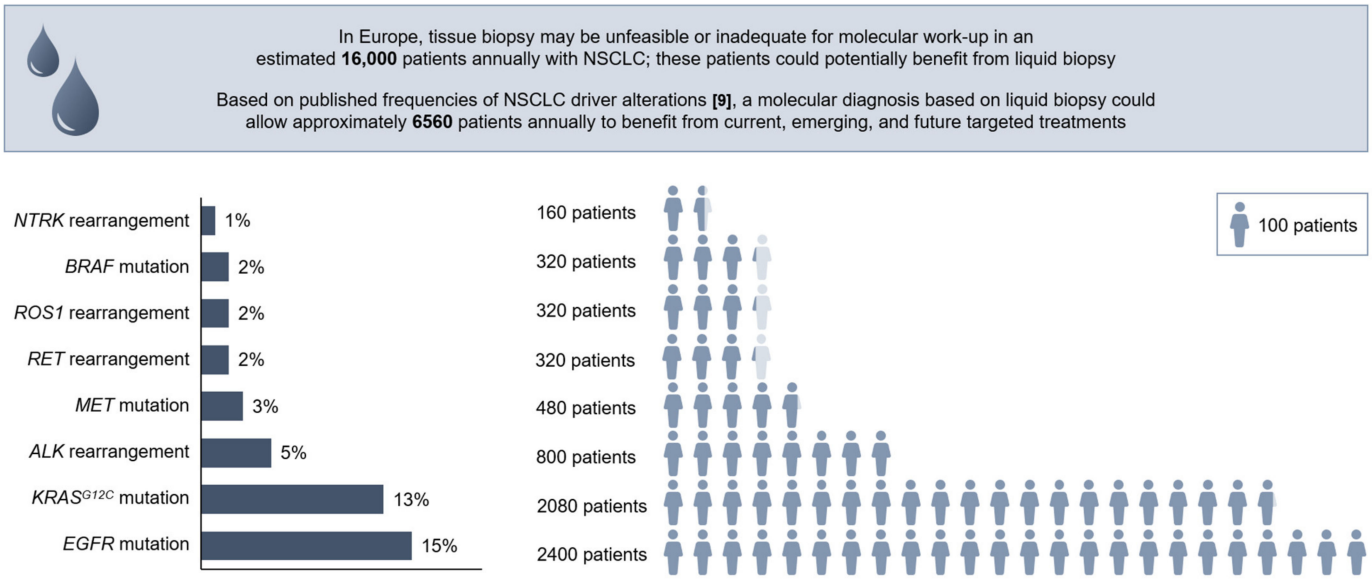

Figure 3. Estimated number of patients in Europe who could potentially benefit from liquid biopsy. Abbreviations: *ALK*, anaplastic lymphoma kinase; *BRAF*, B-Raf proto-oncogene; *EGFR*, epidermal growth factor receptor; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *MET*, hepatocyte growth factor receptor; NSCLC, non-small cell lung cancer; *NTRK*, neurotrophic tyrosine receptor kinase; *RET*, rearranged during transfection; *ROS1*, ROS proto-oncogene 1.

9. What Might Be Next for Liquid Biopsy in NSCLC? An Expert Opinion

Liquid biopsy can clearly complement existing tissue biomarker testing to increase the number of patients with molecular diagnosis, thereby identifying more patients who could benefit from first-line targeted treatment. Liquid biopsy may also help identify patients for appropriate second-line targeted therapy either through detection of circulating markers of resistance or in patients who did not receive frontline biomarker testing. Overall, there are numerous potential applications of liquid biopsy that may provide additional benefit for patients with NSCLC; prospective clinical trials will be required to determine the feasibility of these strategies.

One such emerging application of liquid biopsy is in early-stage NSCLC. Historically, radiological screening for lung cancer in asymptomatic, high-risk individuals has proven effective in reducing mortality, but limitations exist due to the substantial proportion of false positives [84]. False positives can result in unnecessary invasive diagnostic biopsies, which carry the risk of complications [84]. Therefore, an approach that combines the high sensitivity of computed tomography scans with the high specificity of liquid biopsy is potentially attractive. Several liquid biopsy platforms have been evaluated, including CancerSEEK (Thrive Earlier Detection, Cambridge, MA, USA) and CAncer Personalized Profiling by deep Sequencing (CAPP-Seq) [85–87]. Early-stage lung cancer is detectable in cfDNA using a genome-wide sequencing approach, albeit with suboptimal sensitivity [85]. CAPP-Seq has been reported to provide 100% sensitivity for detecting stage II–IV NSCLC but lower sensitivity (50%) for stage I disease. This method could also distinguish treatment-related imaging changes and MRD [86]. Notably, a systematic review conducted by the UK Early Cancer Detection Consortium highlighted the need to standardise sample size, design, and testing procedures in liquid biopsy studies prior to incorporation into screening programmes [88]. An elevated risk of false negatives is associated with liquid biopsy-based detection of early-stage disease since small, localised tumours may not be associated with sufficient levels of tumour DNA shedding to be detectable.

As the persistence of ctDNA following radical treatment correlates with the persistence of MRD [89], liquid biopsy could potentially be used for detection of MRD in NSCLC. Early detection of MRD (i.e., compared with standard radiological procedures) is valuable from a prognostic perspective, as it can identify patients who are likely to experience lung cancer recurrence and permit appropriate management, such as selection for adjuvant therapy [89,90].

While the use of liquid biopsy in early-stage NSCLC could potentially improve treatment outcomes, the prevention of cancer development is even more desirable. Accordingly, liquid biopsy is likely to play an important role in cancer interception—the identification of biomarkers associated with developing cancer and subsequent implementation of therapeutic strategies to prevent the development of cancer (recently reviewed by Serrano and colleagues [91]).

Liquid biopsy may also permit detection of early response to specific targeted therapies through evaluation of plasma clearance prior to tumour assessment. For example, the plasma mutational load of *EGFR*-activating mutations was shown to inversely correlate with outcome measures in patients treated with osimertinib, whereby a trend towards improved outcomes was observed in patients with clearance of plasma mutations during treatment with osimertinib [92].

Given the potential application of liquid biopsy for MRD monitoring, there is sufficient evidence supporting the routine inclusion of liquid biopsy in NSCLC trials (phase 1–3); indeed, this is beginning to manifest accordingly. For example, analysis of cfDNA was conducted in a subset of patients in a recent phase 2 study of tepotinib in NSCLC with *MET* exon 14 skipping mutations; 34 patients (67%) achieved a molecular cfDNA response, of whom 24 (71%) had a radiological response by independent review [57]. Furthermore, the pragmatic inclusion of liquid biopsy in clinical trials would also provide evidence to support its future reimbursement.

A more experimental application of liquid biopsy, for which there is emerging literature evidence, lies in patient selection for treatment with immunotherapy (and combinations of therapy to overcome adaptive resistance). One study has reported strong correlation in the number of predicted neoantigens detected using cfDNA versus tissue biopsies [93], while another showed moderate correlation between plasma and tissue tumour mutational burden [94]. A further study indicated that PD-L1 mRNA may be detected and quantitated in ctRNA [95].

In terms of future technological developments, we envisage an increasing role for customised targeted liquid biopsy panels, which permit the specific and highly sensitive detection of ctDNA mutations relevant for NSCLC. Examples include Tagged-Amplicon deep sequencing (TAM-Seq; Inivata, Little Abington, UK) [96], Safe-Sequencing System (Safe-SeqS; Sysmex, Hamburg, Germany) [97], CAPP-Seq [86], Avenio (based on CAPP-Seq technology; Roche, Indianapolis, IN, USA) [98], LiquidPlex^{TM} (ArcherDX, Boulder, CO, USA) [99], and Ion Torrent (Thermofisher, Waltham, MA, USA) [100]. Ion torrent (mentioned in the IASLC statement paper [42]), Avenio (research use only), Safe-SeqS, and LiquidPlex^{TM} are available as commercial kits, while other companies (Guardant Health, Foundation Medicine, Tempus, and Caris) offer centralised liquid biopsy services. The increased adoption of NGS-based panels may pose challenges to health systems around the world due to the requirements that come with management of large and complex datasets; improved systems to securely manage patient data will be required.

Taken together, the evidence discussed herein indicates that liquid biopsy represents an important emerging diagnostic modality to help ensure optimal care of patients with NSCLC. The rapid advancement in the number of approved targeted therapies for NSCLC coupled with the evolving complexity of resistance mechanisms and potential for combination therapy is likely to amplify the clinical need for liquid biopsy-based disease monitoring. We have provided an estimate of the immediate clinical benefit that broader adoption of liquid biopsy may bring to patients with NSCLC who do not currently receive biomarker testing due to inadequate tumour biopsy material or because they are ineligible for invasive tissue biopsy. Ultimately, the complementary use of liquid and tissue biopsy aims to ensure that all patients with NSCLC receive rapid initial molecular diagnosis and appropriate targeted treatment and that tumour evolution can be monitored to permit appropriate adaptation of therapy on relapse.

Author Contributions: Conceptualisation, J.K.; writing—original draft preparation, U.M., M.T. (Marcello Tiseo), A.V., J.K., M.J.S. and M.T. (Markus Tiemann); writing—review and editing, U.M., M.T. (Marcello Tiseo), A.V., J.K., M.J.S. and M.T. (Markus Tiemann). All authors have read and agreed to the published version of the manuscript.

Funding: Medical writing for this manuscript was funded by Amgen (Europe) GmbH (Rotkreuz, Switzerland).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable. No new data were created or analysed in this article.

Acknowledgments: Medical writing support, including development of a draft outline and subsequent drafts in consultation with the authors, assembling tables and figures, collating author comments, copyediting, fact checking, and referencing, was provided by Emma McConnell, CMPP, and David Evans, at Aspire Scientific Limited (Bollington, UK). We would also like to acknowledge and thank the patients involved in the various studies we have cited who are helping to advance the treatment of NSCLC for future patients.

Conflicts of Interest: U.M. reports personal fees (for service in a speaker bureau and as an advisor) from Amgen, AstraZeneca, Boehringer Ingelheim, Diaceutics, Eli Lilly, Merck, MSD, Roche, and Thermofisher. M.Tiseo has received personal fees (for service in a speaker bureau and as an advisor) from AstraZeneca, Bristol Myers Squibb, Boehringer Ingelheim, Eli Lilly, MSD, Novartis, Otsuka,

Pierre Fabre, Pfizer, Roche, and Takeda; and research funding from AstraZeneca and Boehringer Ingelheim. A.V. has received personal fees (for service as an advisor) from Bristol Myers Squibb, Guardant Health, Merck, Novartis, Roche, and Sysmex; and research funding from Bristol Myers Squibb, Cellestia Biotech, Debio, Novartis, Roche, and Sysmex. J.K. is employed by Amgen and has stocks/shares in Amgen. M.J.S. has received honoraria from Astellas and GSK. M. Tiemann reports honoraria from AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Merck, Pfizer, Roche, and Takeda.

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