Review Article

Current and future applications of liquid biopsy in non-small-cell lung cancer—a narrative review

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Background and Objective: Lung cancer remains the leading cause of cancer-related mortality and constitutes a significant societal burden. Recent advancements in targeted therapies and immunotherapy have considerably broadened therapeutic options in lung cancer, particularly in non-small-cell lung cancer (NSCLC). However, these novel methods necessitate sophisticated molecular diagnostics. Liquid biopsy, which refers to the cytological and molecular analysis of cancer markers shed by the tumor into the body fluids, may offer an attractive diagnostic tool at the individual patient level. This approach is particularly relevant for lung cancer, as the anatomical location of tumor lesions frequently makes them inaccessible for tissue biopsy. Apart from minimal invasiveness, the major advantages of liquid biopsy include better reflection of the tumor clonal heterogeneity (spatial heterogeneity), the possibility of sequential sampling, and real-time monitoring of tumor load and its evolving mutational status (temporal heterogeneity).

Methods: This article reviews the available data in this field, current applications, and future perspectives in accordance with the Narrative Review reporting rules.

Key Content and Findings: We discuss the most used approaches, i.e., circulating DNA and tumor cells, but also emerging liquid biopsy techniques, such as plasma DNA methylation, plasma metabolites and RNA, extracellular vesicles, and tumor-educated platelets in NSCLC. Finally, we highlight the current limitations of liquid biopsy techniques hampering their clinical applications.

Conclusions: Due to their advantages, liquid biopsy-based approaches have recently gained immense interest in oncology. Potential applications of this method include early detection, informing precision medicine-based individualized treatment, and real-time monitoring of disease evolution and treatment. The development of next-generation sequencing has vastly extended genetic profiling, thus enabling better identification of druggable alterations. However, the clinical application of liquid biopsy techniques is still limited due to their suboptimal specificity and sensitivity, lack of standardization, and relatively high costs. Addressing these issues may allow further integration of liquid biopsies in the routine clinical setting, thus making a profound and permanent change in NSCLC management.

Keywords: Circulating tumor DNA (ctDNA); circulating tumor cells (CTC); liquid biopsy; non-small-cell lung cancer (NSCLC)

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Introduction

With an estimated 1.8 million deaths accounting for 18% of global cancer mortality, lung cancer remains the leading cause of cancer death (1). During the past two decades, the application of targeted therapies and, more recently, immune checkpoint inhibitors (ICIs) has resulted in a spectacular improvement in treatment efficacy in lung cancer. However, this progress mainly refers to non-small-cell lung cancer (NSCLC). Modern management of NSCLC using precision medicine methods necessitates sophisticated molecular diagnostics. Genetic testing is currently a routine procedure for many therapeutic targets, such as activating mutations in epidermal growth factor receptor (*EGFR*), *HER2*, *BRAF*, *KRAS*, *MET* exon 14 skipping; rearrangements of anaplastic lymphoma kinase (*ALK*), *NTRK*, *RET*, and *ROS* proto-oncogene1 (*ROS1*) (2,3). New-generation assays also allow quantification of tumor mutational burden (TMB), which is associated with response to ICIs (4,5).

NSCLC presents a large intratumor heterogeneity and genomic instability. Acquiring molecular changes and treatment-induced clonal selection leads to genetic evolution and resistance to systemic therapies (6-8).

Tissue biopsy remains the gold standard in pathological NSCLC diagnosis, tissue genotyping, and informing treatment (9-11). However, the limitation of this approach lies in its inability to address the clonal heterogeneity of this malignancy (11). More importantly, the increasing number of treatment-guiding biomarkers usually necessitates obtaining many tumor biopsies and repeated sampling to tailor subsequent treatments. This is often not feasible or risky due to the anatomical location of the primary or metastatic lesions or the worsening general condition of the patient (12).

Liquid biopsy, which refers to the cytological and molecular analysis of cancer markers shed by the tumor into the body fluids, is a minimally invasive and easily repeatable test. The most common source for liquid biopsy is blood, as it provides the largest array of biological analytes, such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), tumor-educated platelets (TEPs), circulating exosomes, DNA methylation, and metabolomic and proteomic markers (*Figure 1*). In addition to convenience, ease of access and minimal invasiveness, the major advantages of liquid biopsy include better reflection of the tumor clonal heterogeneity (spatial heterogeneity), the possibility of sequential sampling, and real-time monitoring of tumor load and its evolving mutational status (temporal heterogeneity). Supplementing the standard tissue evaluation with liquid biopsy was also shown to save system costs and increase the number of patients administered appropriate targeted therapies (13-15). Finally, a liquid biopsy may significantly shorten turnaround times at primary diagnosis and at progression (16-18).

The first clinical application of liquid biopsy techniques in NSCLC included testing for EGFR mutational status (19,20). Within the past decade, liquid biopsy-based approaches have gained immense interest in oncology, manifested by thousands of publications. A milestone in their widespread use was the development of high-throughput sequencing methods, in particular next-generation sequencing (NGS). This method has considerably broadened genetic profiling, thereby allowing for better identification of druggable alterations and their evolution throughout the disease course (21).

Potential clinical applications of liquid biopsy in NSCLC include early detection, informing precision medicine-based individualized treatment, and real-time monitoring of disease evolution and treatment (21).

Thus far, the only circulating NSCLC biomarker in routine clinical use is ctDNA, which was approved in 2016 for selecting patients for targeted therapies (21,22). Other liquid biopsy sources are in various phases of clinical development. This review summarizes current knowledge on liquid biopsy in NSCLC and its clinical applications, with a particular focus on emerging techniques. We present the following article in accordance with the Narrative Review reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-742/rc).

Methods

In this review, PubMed/MEDLINE, Embase and Google Scholar databases were searched between the date of database inception and December 30, 2022. Only articles in English were evaluated. Details are listed in *Table 1*. The figures were created using BioRender.com.

ctDNA

In 1948, Mandel and Metais first described the presence of short DNA fragments (termed cell-free DNA, cfDNA) in the blood (23). In healthy individuals, they mainly originate from the hematopoietic cells and reflect their clonal heterogeneity (24,25). In 1977, Leon *et al.* reported an increase in cfDNA content in cancer patients (26), which

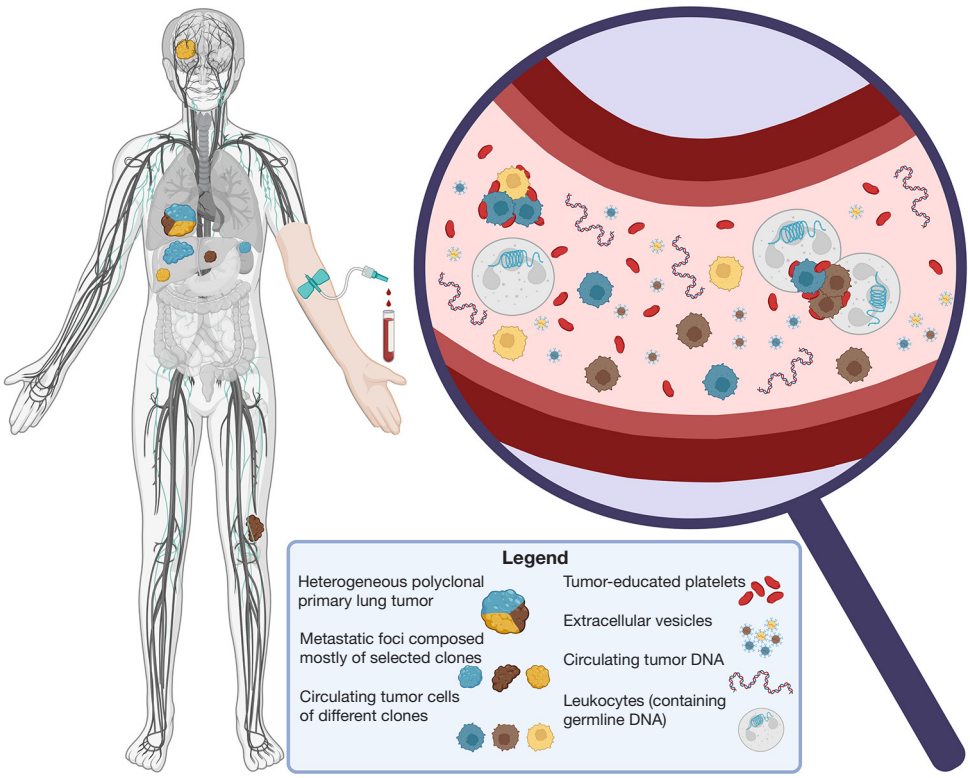

Figure 1 Liquid biopsy materials in NSCLC. Tumor derivates include circulating tumor DNA, circulating tumor cells, extracellular vesicles, and tumor-educated platelets. NSCLC, non-small-cell lung cancer.

Table 1 The search strategy summary

Items	Specification
Date of search	Last update 01/01/2023
Databases and other sources searched	PubMed/MEDLINE, Embase, Google Scholar
Search terms used	"non-small-cell lung cancer", "liquid biopsy", "circulating DNA" and "circulating tumour cells"
Timeframe	Databases were searched between the date of database inception and December 30, 2022
Inclusion and exclusion criteria	Only articles in English were evaluated, only full texts were evaluated
Selection process	Each step of the study was conducted by two independent researchers (BT and MB) with disputes resolved by discussion

was later attributed to neoplastic cell death (27). However, the interest in ctDNA remained modest until it boomed following the development of PCR-based techniques. Importantly, ctDNA has a very short half-life, thus making it an ideal biomarker for disease monitoring (28,29).

The pre-analytical factors that may influence the accuracy of ctDNA evaluation remain poorly described. One aspect of the general consensus is the use of plasma over serum samples to prevent unnecessary leukocyte lysis and, thus, genomic DNA contamination (30,31). Among the numerous available cfDNA extraction methods, neither has been proven superior with significant interlaboratory variability (32,33). In addition, the timing of sample collection is crucial, as numerous factors (including physical exercise, inflammatory conditions, concurrent treatment, and time of the day) may affect cfDNA levels (11,34). Therefore, no unanimously agreed protocol for sample collection has not yet been defined, and procedural

uniformity within a study becomes vital (11). Another important aspect of ctDNA testing is clonal hematopoiesis of indeterminate potential (CHIP), i.e., somatic gene variants, common in aging human hematopoietic stem cells. CHIP is generally defined by any variant allele frequency (VAF) of at least 2% (35). Some of these variants are relatively frequent in solid malignancies. As these variants are also detectable in cfDNA, they are a relevant source of biological noise in liquid profiling. Hence, to avoid false positive results, it is advisable to couple cfDNA testing with whole blood sequencing as a control (36). This might however impact time- and cost-effectiveness. Hence, novel approaches involving machine learning are currently under investigation (37).

It is also essential to distinguish between tumor-informed and tumor-agnostic strategies. The former involves tumor specimen sequencing to create a tumor-specific signature. Identified aberrations may then be tracked using polymerase chain reaction (PCR)-based techniques or NGS panels. Focusing on already-known alterations allows for deeper coverage, making these assays more sensitive. A tumor-informed probe serving as an internal control also increases specificity by reducing background noise from not tumor-derived clonal populations, e.g., CHIP. However, this approach has a long turnaround time and requires high tissue quality. In turn, a tumor-agnostic strategy works across various cancer types sharing the same targetable abnormality. This approach is faster, cheaper, and does not necessitate sequencing the primary tumor, but at a price of lower sensitivity (38).

In metastatic NSCLC, ctDNA testing already plays a vital role in clinical practice, allowing the detection of tumor-derived somatic aberrations from plasma to inform treatment decisions (39). Although tissue is still the gold standard for the initial NSCLC genotyping, complementary ctDNA testing may provide valuable insights into possible targets missed by tissue biopsy. Liquid biopsy may have particular value for patients with a discordant clinical history or a high probability of intratumor heterogeneity. Complementary ctDNA testing may also be helpful when tumor biopsy is difficult, or the sample amount is insufficient (11,40). This approach is now endorsed by the European Medicines Agency (EMA), the International Association for the Study of Lung Cancer, and the National Comprehensive Cancer Network (11,39,41,42). Plasma and tissue genotyping seem complementary, with 20% of targetable variants detected in blood but not in tissue (43). In addition, plasma-based assays allow for a shorter turnaround time (44).

In 2020, the US Food and Drug Administration (FDA) and EMA approved the use of two ctDNA assays, Guardant360 and FoundationOne Liquid CDx, to identify genomic alterations in patients with advanced-stage solid malignancies (45,46). Both are hybrid capture-based NGS assays that detect variants in 73 and 311 genes, respectively (47,48). Detecting *EGFR* variants in ctDNA from peripheral blood is already widely used (*Figure 2*). Two platforms, the cobas® EGFR mutation test v2 (Roche, Basel, Switzerland) and the TheraScreen EGFR RGQ PCR Kit (Qiagen, Hilden, Germany), have been approved by both the EMA and FDA for the testing of *EGFR* variants in liquid biopsies (49). Many studies have shown high concordance in EGFR status between ctDNA and tissue biopsies (50,51). The role of liquid biopsy is particularly compelling at disease progression, as it may allow tracking mechanisms of resistance. For example, in *EGFR*-mutated NSCLC, approximately 30% of T790M-positive tissues are missed by plasma, and a similar proportion of cfDNA T790M-positive results would test negative in tissues, making these two approaches complementary (52). This is of particular importance because the clinical gain from osimertinib, a third-generation EGFR tyrosine kinase inhibitor, has been demonstrated in both plasma-positive and tissue-positive groups (52). The feasibility of using ctDNA as a surrogate for tumor biopsy was shown in the EURTAC trial, which provided evidence that progression-free survival (PFS), overall survival (OS), and response to therapy are associated with the type of *EGFR* variants in ctDNA (53). Unlike single variants, molecular aberrations, such as fusions, copy number variations, and some focal amplifications, appear more difficult to detect in blood than in tissue (54). Nevertheless, genomic profiling of ctDNA has also been demonstrated to be a reliable and useful method for genetic profiling and monitoring tumor evolution in *ALK*-rearranged NSCLCs (55-59). Two ctDNA assays, FoundationOne® Liquid CDx and Guardant360® CDx (Guardant Health), have been approved by the FDA for testing this aberration and found clinical applications (40).

High levels of ctDNA are also linked to worse survival; hence, ctDNA can contribute to estimating prognosis (*Figure 2*) (60). In addition, the persistence of ctDNA after surgical treatment of early-stage NSCLC may be indicative of disease recurrence and can identify patients for further intervention (61). ctDNA detection in advanced NSCLC is reasonably reliable, although sensitivity in early-stage tumors remains low, usually not exceeding

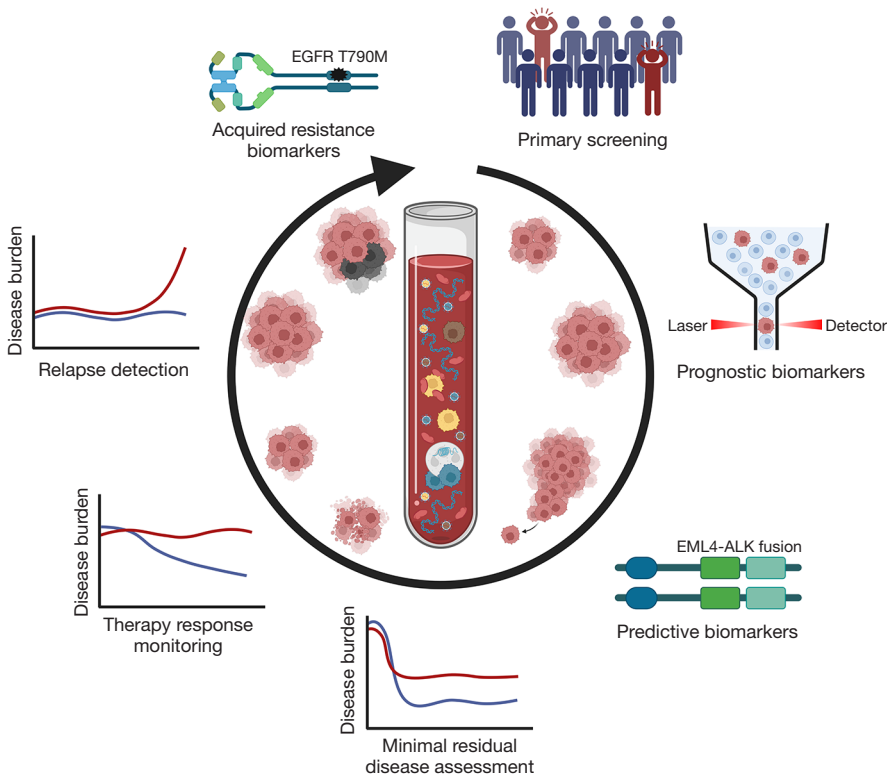

Figure 2 Overview of current and potential applications of liquid biopsy in non-small-cell lung cancer. EGFR, epidermal growth factor receptor; EML4, echinoderm microtubule-associated protein-like 4; ALK, anaplastic lymphoma kinase.

50% (62,63). Nevertheless, plasma ctDNA could probably be a valuable adjunct to other screening methods, such as low-dose computed tomography (LDCT), and is an approach under evaluation in the ongoing SUMMIT study (NCT03934866).

While approved assays can be applied to plasma samples from patients with advanced NSCLC, they do not perform very well for minimal residual disease (MRD) monitoring. With increasing attention and progress toward the earlier detection of solid tumors, the relevance of detecting MRD and early recurrence may transform the optimal treatment paradigm through more timely interventions (*Figures 2,3*). The concept of MRD has been introduced in hematology, where the presence of residual malignant cells following treatment was shown to be associated with poorer prognosis and earlier relapse (64). Nowadays, MRD-guided therapy has become widely introduced in hematology, and MRD itself is used as a surrogate efficacy-response biomarker to accelerate drug development (65). This approach has also been successfully tested in stage II colon cancer, where the evaluation of ctDNA after surgery led to reduced adjuvant chemotherapy use without compromising recurrence-free survival (66). Similar to MRD testing in hematology, ctDNA may have utility for residual disease detection following curative-intent treatment and during the surveillance period for relapse (*Table 2*). Although commercially available assays have not yet achieved regulatory approval, several trials evaluating the role of ctDNA in the personalized treatment of patients with NSCLC are ongoing (*Table 2*).

As ICIs become increasingly common in the treatment of NSCLC, the potential of ctDNA to guide and follow the response to immune therapy is also being evaluated (*Figure 2*) (72). Good concordance of ctDNA NGS with tissue NGS enables the estimation of TMB (73). A high blood-based TMB (bTMB) was associated with improved response to ICIs in the POPLAR and OAK trials (73). However, the test outcome is highly dependent on the gene panel (73). Recently, B-F1RST, an open-label, phase 2 trial that evaluated bTMB as a predictive biomarker for first-line atezolizumab monotherapy in locally advanced or metastatic stage IIIB–IVB NSCLC, showed that bTMB ≥16 was associated with longer OS (74). Additionally, the overall

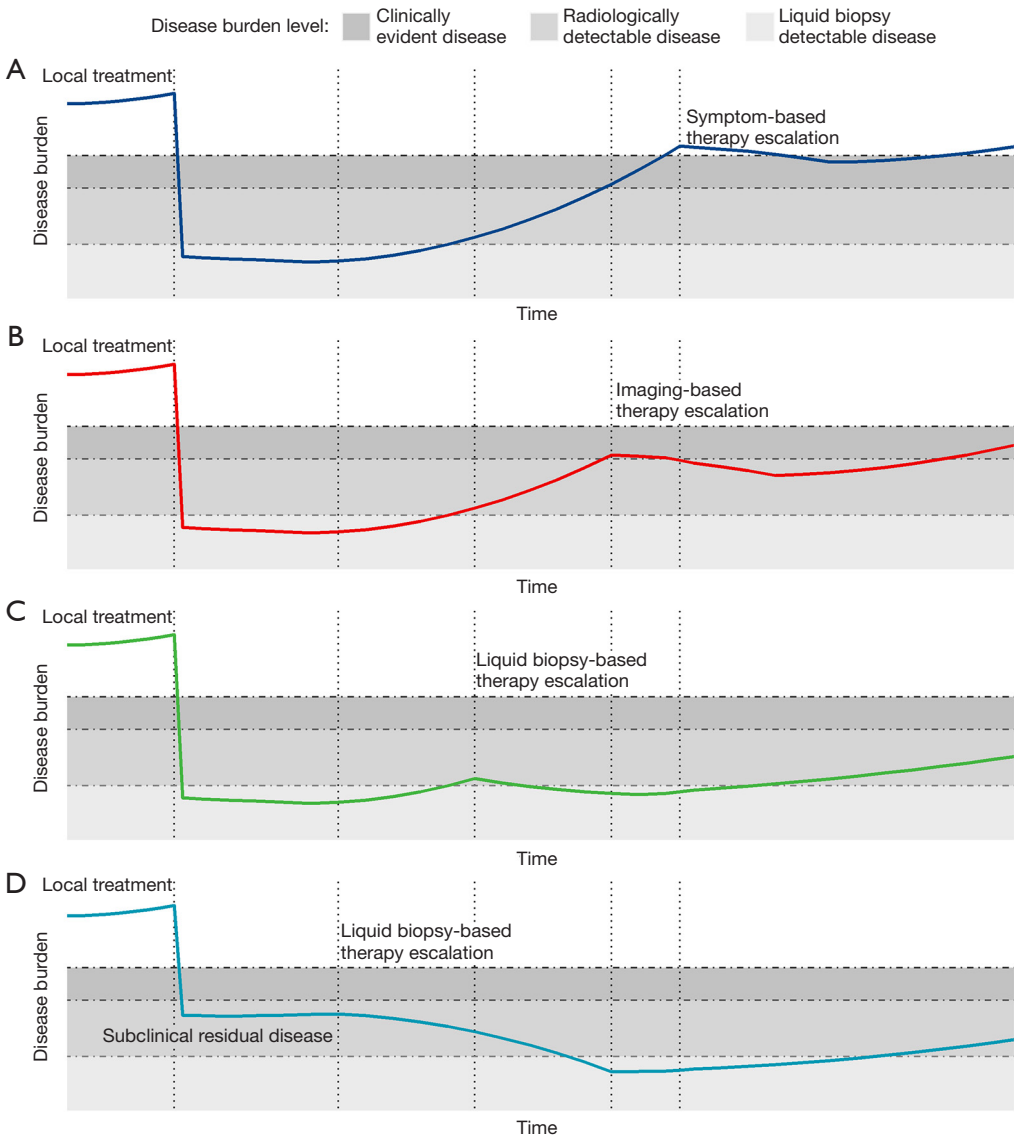

Figure 3 Hypothetical disease control available when guiding treatment using the following approaches: (A) physical examination, (B) imaging, (C) liquid biopsy-based assays, and (D) liquid biopsy-based screening for minimal residual disease after curative local treatment.

response rate (ORR) improved with increasing bTMB cutoffs.

Although higher TMB is generally associated with clinical benefit from ICIs, the clinically relevant bTMB cutoff values are lacking. Clinical trials have measured TMB using Whole Exome Sequencing (WES) or the FoundationOne CDx assay. Later on, several targeted panels appeared on the market, providing TMB estimates in a time- and cost-effective manner (75). A recent initiative of the Friends of Cancer Research TMB Harmonization Project showed substantial variability between assays. The same group developed and made publicly available a software tool that could promote reproducibility and comparability across various assays (76).

ctDNA has also been proposed as an ICI monitoring tool to discriminate between pseudo-progression and true progression (77). Rapid decreases in ctDNA levels after initiating first-line pembrolizumab with or without chemotherapy were associated with significantly higher response rates and longer PFS and OS (78).

The optimal post-treatment time point for ctDNA MRD assessment remains unclear. Addressing this question is of

Table 2 Studies investigating the role of ctDNA in MRD evaluation and surveillance in NSCLC patients

Study	N	Stage	Treatment	Sensitivity, %	Specificity, %	Assay
ctDNA performance to detect MRD						
Chaudhuri *et al.*, 2017 (67)	32	IB–IIIB	CRT or RT and/or surgery ± CHT	94	100	CAPP-Seq
Abbosh *et al.*, 2017 (68)	24	IA–IIIB	Surgery ± CHT ± PORT	36	90	Signatera
Chen *et al.*, 2019 (69)	25	IIB–IIIB	Surgery ± CHT ± PORT	44	88	cSMART
Zviran *et al.*, 2020 (70)	22	IA–III	Surgery ± CHT and RT	100	71	MRDetect
ctDNA performance to detect disease in the surveillance setting						
Chaudhuri *et al.*, 2017 (67)	37	IB–IIIB	CRT or RT and/or surgery ± CHT	100	100	CAPP-Seq
Abbosh *et al.*, 2017 (68)	24	IA–IIIB	Surgery ± CHT	93	70	Signatera
Abbosh *et al.*, 2020 (71)	78	I–III	Surgery ± CHT	82	96	ArcherDx

ctDNA, circulating tumor DNA; MRD, minimal residual disease; NSCLC, non-small-cell lung cancer; CRT, chemoradiotherapy; RT, radiotherapy; CHT, chemotherapy; PORT, postoperative radiotherapy.

utmost importance and requires initiatives to harmonize clinical trials measuring ctDNA. An example of such an initiative is the ctMoniTR harmonization effort of post-ICI ctDNA data in advanced NSCLC. The evidence from prospective studies is currently scarce [NILE trial, NCT03615443 (44)], and most current data on the clinical utility of plasma ctDNA genotyping come from retrospective observational studies (79). Also, it is still unknown whether an increase in plasma ctDNA levels alone should lead to treatment modification. The APPLE-EORTC trial, a randomized, open-label, multicenter, 3-arm, phase II study of advanced EGFR-positive NSCLC, addressed this issue (80,81). A recent study showed that serial monitoring of ctDNA T790M status by Cobas v2.0 PCR test in patients with advanced EGFR mutant-NSCLC treated with first-generation EGFR inhibitors is feasible and allows for an earlier switch to second-line EGFR inhibition (81). However, the study evaluated only T790M-driven acquired resistance; therefore, extrapolation of its results to other clinical scenarios is disputable. More studies with appropriate sample size and multiple post-treatment time points are needed to improve the understanding of ctDNA MRD and its predictive role in NSCLC. A summary of major ongoing trials is presented in *Table 3*.

CTCs

CTCs play an important role in metastasis formation and represent an intermediate stage of this process (82). Despite their rare occurrence (approximately 1–10 per 10 mL of blood), they are accessible through a simple and non-invasive sampling of body fluids. Only approximately 0.02% of CTCs can survive the adverse environment, which includes continuous mobility, immune attack, mechanical shear forces, and oxidative stress (83). CTCs may occur as single cells, clusters of several cells, or circulating tumor microemboli consisting of huge numbers of cells aggregated together with other cells, including hematopoietic cells, platelets, and stromal cells (84). The clustered forms are more stable in the bloodstream and have a greater metastatic capacity than single CTCs (85). The most analyzed source of CTCs is pulmonary vein blood because their counts are higher there than in peripheral vessels (86,87). Numerous methods of CTC detection can include those based on microfluidics and the physical properties of the cells, in particular their size, the functional or immune assays. The most frequently applied approaches usually employ epithelial cell enrichment (with EpCAM antibodies) (88), leukocyte depletion (with CD45 antibodies) (89), or both (90). In contrast to ctDNA, CTC analysis necessitates sample collection and processing specifically matched to the isolation method and downstream applications. Major characteristics and the comparison between ctDNA/cfDNA and CTCs are presented in *Table 4*.

CTCs as a screening tool

Blood-based biomarkers may act as a standalone screening tool (*Figure 2*) or in addition to other screening methods, such as LDCT in NSCLC (91). In 2006, a study using a

Table 3 Ongoing clinical trials investigating the role of ctDNA in personalized treatment of NSCLC

Trial acronym/ NCT #	Phase	NSCLC stage	Primary endpoint	Primary treatment	Moment of ctDNA measurement	ctDNA (+) intervention	ctDNA (–) intervention	Planned enrollment	Recruitment status	Type of assay
MERMAID-1 (NCT04385368)	III	II–III	DFS in MRD + analysis set	Surgery	After surgery	Durvalumab + SoC CHT *vs*. placebo + SoC CHT	N/A	332	Recruiting	ArcherDx
MERMAID-2 (NCT04642469)	III	II–III	DFS in the PD-L1 TC ≥1% analysis set	Surgery +/– neoadjuvant or adjuvant treatment	Surgery +/– neoadjuvant or adjuvant treatment	Durvalumab *vs*. placebo	N/A	284	Active, not recruiting	ArcherDx
NCT04585490	III	III	Change in ctDNA level following CHT	CRT	After CRT	4 cycles of CHT (platinum-based doublet) + durvalumab (1,500 mg IV every 21 days for 1 year)	SoC durvalumab (10 mg/kg every 2 weeks, or equivalent, for 1 year)	48	Recruiting	Avenio
NCT04585477	II	I–III	Decrease in ctDNA level	Surgery or definitive SBRT	After surgery or SBRT	12 cycles of durvalumab	SoC and no treatment	80	Recruiting	Avenio
SCION (NCT04944173)	II	I–IIA	ORR at 18 months	SBRT + 4 cycles of durvalumab	After SBRT + 4 cycles durvalumab	Additional 8 cycles of durvalumab	No further treatment	94	Not yet recruiting	Avenio

ctDNA, circulating tumor DNA; NSCLC, non-small-cell lung cancer; DFS, disease-free survival; MRD, minimal residual disease; SoC, standard of care; CHT, chemotherapy; PD-L1, programmed death ligand 1; TC, tumor cell; N/A, not applicable; CRT, chemoradiotherapy; IV, intravenous; SBRT, stereotactic body radiotherapy; ORR, overall response rate.

17-gene array found CTCs in 90% of NSCLC patients at different disease stages and in 6% of healthy controls (92). However, the performance of this tool was associated with cancer stage and was the lowest in stage I disease. Other studies using the folate receptor transcript as a marker to identify CTCs have shown promising sensitivity and specificity (93,94). However, this approach was also hampered by lower performance in the early stages of NSCLC. A study including patients with chronic obstructive pulmonary disease (COPD), which often coexists with NSCLC, identified CTCs in 3% (5 out of 168 patients) of the group (95). During follow-up with annual CT imaging, lung nodules were found one to four years after CTC detection, and CTCs were undetectable after the surgery. Another study from China showed the *in vivo* CTC detection strategy to be characterized by 53% sensitivity and 90% specificity for diagnosing early-stage NSCLC (96). However, since the evaluated cohort was small, the study was underpowered and inconclusive. On the other hand, a prospective study done on 614 COPD patients treated in 21 French university centers showed only 26% sensitivity of CTC detection for NSCLC, bringing into question the utility of this method in NSCLC screening (97).

CTCs for the evaluation of cancer prognosis

The prognostic value of CTCs in NSCLC has been extensively studied. As mentioned earlier, the number of CTCs generally correlates with the NSCLC burden, and the detection rate varies greatly from 15% to 100% (98-110). Hence, the utility of CTC assessment is highest in more advanced tumors. A decrease in CTC counts after treatment may indicate cancer remission, while an increase may herald cancer progression. Changing CTC counts have been associated with shorter disease-free survival (DFS), PFS, and OS; however, the optimal threshold of detectable CTCs remains to be determined (111-117).

A prospective study including stage IV NSCLC identified CTCs in most subjects (118). The absolute CTC counts were not associated with the prognosis, whereas changes in CTC counts were predictive of OS. The largest study of CTCs in advanced NSCLC included 550 patients

Table 4 Characteristics and comparison of ctDNA/cfDNA and CTCs

Feature	ctDNA/cfDNA	CTCs
Collection	Easier	More complex
Isolation	Easier	More complex
Cell culture	Impossible	Possible
Therapy monitoring	Changes in levels may reflect response/resistance/relapse	
Genomic analyses	Possible	Possible
Transcriptomic analyses	Impossible	Possible
Protein analyses	Impossible	Possible
Functional analyses	Impossible	Possible
Methylation analyses	Possible	Possible
FISH analyses	Impossible	Possible
Single-cell analyses	Impossible	Possible
Chromosomal analyses	Impossible	Possible
Challenges	Potential affecting by treatment- or stress-induced cell death	CTC heterogeneity
	Potential confounding by CHIP	
	Small levels or quantities in circulation	
	Sampling bias	
	Low signal-to-noise ratio in early-stage disease	
Cost	Several hundred to several thousand USD (depending on the panel used)	Several hundred dollars to one thousand USD (CellSearch®)

ctDNA, circulating tumor DNA; cfDNA, cell-free DNA; CTCs, circulating tumor cells; FISH, fluorescent in situ hybridization; CHIP, clonal hematopoiesis of indeterminate potential; USD, United States dollar.

through multicenter European collaboration (119). This study confirmed the independent prognostic value of CTCs for PFS and OS. The detection of CTCs was associated with worse PFS (≥2 CTCs: HR =1.72, P<0.001; ≥5 CTCs: HR =2.21, P<0.001) and OS (≥2 CTCs: HR =2.18, P<0.001; ≥5 CTCs: HR =2.75, P<0.001). Importantly, CTC counts added to clinicopathological predictive models significantly improved their performance. In addition, the presence of circulating tumor microemboli coupled with clinical and imaging data significantly improved diagnostic accuracy in early-stage NSCLC (120). Similar findings were reported in small-cell lung cancer (SCLC). The analysis of CTCs collected from patients enrolled within CONVERT trial showed that the threshold ≥15 CTCs was associated with worse OS independent of all other factors (26.7 months in the group <15 CTCs and 5.9 months in the group ≥15 CTC) (121).

CTCs for guiding treatment

A study using telomerase-based CTC assay in patients with early-stage NSCLC managed with stereotactic body radiotherapy (SBRT) showed that higher pretreatment CTCs (≥5 cells/mL) and persistent CTCs after SBRT were associated with increased risk of regional and distant recurrence (122). This CTC assay may identify subsets of patients who can maximally benefit from adjuvant systemic therapy and enables early detection of recurrence or progression.

CTCs may also be useful in monitoring patients harboring oncogenic driver variants, such as *EGFR*-activating mutations or *ALK* fusion rearrangements. In 2008, Maheswaran *et al.* reported the detection of *EGFR* mutations in CTCs isolated from 27 NSCLC patients (123). The clinical utility of single CTC sequencing in *ALK*-rearranged NSCLCs was

demonstrated by Pailler *et al.* (124). The receptor tyrosine kinase-KRAS pathway (*EGFR*, *KRAS*, *BRAF* genes) and TP53 pathways were recurrently mutated in the CTCs of crizotinib-resistant patients. Another study from this group identified *ROS1* rearrangements in the CTCs of four NSCLC patients (125). Gorges *et al.* identified *KRAS* variants in CTCs that are potentially relevant to treatment decisions (126).

The role of CTCs has also been evaluated in the real-time monitoring of immune cell activation. The presence of PD-L1(+) CTCs was associated with poor prognosis in patients with advanced NSCLC (127). However, the data regarding concordance between tumor tissue and CTCs are conflicting (127,128). Most of these studies were small, making their results inconclusive.

Although monitoring for CTCs may provide predictive information, the lack of a standardized methodology for CTC enrichment and detection has hampered its uptake by the clinical community. Ongoing initiatives within the framework of collaborative groups, such as CANCER-ID, a European consortium of 38 partners from 13 countries, may better define the clinical utility of liquid biopsies (129).

Emerging technologies and strategies for liquid biopsy

Given the suboptimal sensitivity of DNA variants detection in cfDNA for the early diagnosis of NSCLC (130), new liquid biopsy methods, e.g., DNA methylation, metabolomic, proteomic or RNA markers, or new marker bio-reservoirs, such as tumor educated platelets (TEPs), are being explored. Plasma metabolites might be particularly useful as companion diagnostics for drugs targeting cancer metabolic vulnerabilities. TEPs are thought to provide a unique pool of markers and have the potential for early cancer detection and monitoring after treatment.

Plasma DNA methylation

The low performance of plasma cfDNA for mutation-based cancer detection is due to the limited number of specific cancer variants, affecting only a handful of genomic locations. Moreover, the amount of ctDNA is relatively low compared with the non-tumor cfDNA, resulting in a low signal-to-noise ratio. These problems are further confounded by the variants arising concomitantly in hematopoietic cells, namely aging-related clonal hematopoiesis variants that may mimic cancer-associated mutations (25,131). In contrast, the cfDNA methylation spans nearly 30 million sites known as CpG sites across the human genome, which enables the specific selection of CpG sites not shared by other cancer locations (132). Cancer-specific methylation patterns have been established and can be inferred from plasma DNA with the advance of bioinformatics methods (133). The large-scale comparisons between cfDNA sequencing and cfDNA whole genome bisulfite sequencing have shown the superior sensitivity of cancer detection of the latter (134).

The recently developed Galleri targeted-methylation multi-cancer early detection (MCED) test showed an overall sensitivity of 52% for detecting malignancy across more than 50 cancer types (135). At 99.5% specificity, the sensitivity for stage I–III cancer detection was 68% in 12 pre-specified cancers and 75% in lung cancer. However, the sensitivity of stage I NSCLC detection was only 22%, compared with 80% and 91% in stages II and III, respectively. The overall accuracy of the primary tumor site determination in true positives was 89%, which is suitable for informing care after the positive screening test result. This test was assessed prospectively in an epidemiological interventional Pathfinder study (136). Healthy individuals with positive test underwent test-directed diagnostic procedures toward a cancer diagnosis. In this single-arm study, the primary endpoints included the time required to establish clinical cancer diagnosis following a positive MCED blood test and the number and types of diagnostic tests used. The test detected a cancer signal in 1.4% of 6,621 individuals 50 years or older not known to have cancer. Cancer was confirmed in 38% and 43% of those with a positive test using earlier and refined versions, respectively. The median time to confirm or exclude a tumor was 79 days among participants with a positive screening test, and 73% of subjects obtained the diagnostic resolution within three months. Overall, adding MCED testing to the standard screening doubled the cancer detection rates. The Galleri test is currently being assessed in England in a prospective trial of 140,000 participants (137).

Recently, genome-wide cfDNA methylation profiling was found to provide specific patterns for SCLC (138). Additionally, the levels of tumor methylation detected in cfDNA correlated with OS. SCLC comprises several molecular subtypes with differential therapeutic vulnerabilities (139). For example, ASCL1 drives a phenotype susceptible to BCL2 apoptosis regulator and δ-like canonical Notch ligand 3 inhibitors, whereas the NEUROD1-driven subtype is sensitive to Aurora kinase

inhibitors (140,141). The cfDNA SCLC-methylation patterns may differentiate between these molecular subtypes, potentially allowing for monitoring the dynamics of the molecular make-up along the disease progression (138).

Plasma metabolites

In multiple cancer types, the cell metabolism is altered to initiate or promote cancerogenesis or support the demands of high proliferation (142). Specific cancer phenotypes characterized by persistent dysregulation of metabolic pathways could be leveraged for cancer diagnosis with liquid biopsy (141). Sensitive detection techniques, such as liquid chromatography-mass spectroscopy (LC-MS), have enabled the interrogation of plasma for a vast array of metabolites (142). Moreover, metabolite addiction typical of certain cancers may result in metabolic vulnerabilities constituting potential targets for therapeutic interventions (143,144). For example, NSCLC with mutational activation of Nrf2, an antioxidant and detoxification transcription factor, becomes dependent on glutamine, which could be exploited therapeutically using glutaminase inhibitors or G6PD inhibitors (145,146). Thus, the plasma metabolites may be considered to be potential predictive markers of response to metabolically targeted anticancer therapies.

In a study of 25 early-stage NSCLC patients, the major serum metabolic alterations included increased levels of ketone bodies and lactate and decreased levels of glucose, lipids, choline phospholipid metabolites, TMAO, and betaine, compared with matched healthy controls (59). The levels of glutamine, glutamate, asparagine, aspartate, tyrosine histidine, cysteine, isoleucine, and leucine were increased in the serum of cancer patients, whereas the levels of tryptophan and methionine were reduced. In another study of 110 NSCLC patients and 43 healthy controls, targeted metabolomic analysis with LC-MS, a specific combination of six metabolic biomarkers enabled discrimination between stage I NSCLC patients and healthy individuals with 98% sensitivity and 100% specificity. An exhaustive summary of studies regarding NSCLC was presented earlier (147).

Cancers of different lineages may utilize some metabolites differently. For example, *KRAS* activation and *Trp53* inactivation result in formation of pancreatic ductal adenocarcinoma and NSCLC. However, despite sharing the same initiating events, the plasma branched-chain amino acid levels are elevated in pancreatic cancer and normal in NSCLC, as its growth relies on branched-chain amino acid metabolism (148). The analysis also revealed distinct metabolic signatures for lung adenocarcinoma and squamous cell carcinoma (149).

Glioma and acute myeloid leukemia cells with IDH1 mutation constitutively produce an oncometabolite D-2-hydroxyglutarate (D-2-HG) with the diminished production of its normal IDH1 product, i.e., α-ketoglutarate (αKG) (150-153). The decreased amounts of αKG in cancer cells release hypoxia-inducible factor 1 (HIF-1), which leads to widespread pro-oncogenic consequences and contributes to the progression of these malignancies. Mutations in the *IDH-1* and *IDH-2* genes occur in majority of malignant gliomas (60–90%), 10–20% of acute myeloid leukemias, and up to approximately 1% of lung adenocarcinomas (154,155). Significantly elevated levels of D-2-HG in cells, tissues, plasma, and urine from cancers with somatic variants in IDH may indicate the presence of respective malignancies. This oncometabolite is present in negligible amounts in non-IDH mutant cells (156). The tissue levels of D-2-HG were reported to be increased in lung adenocarcinoma compared with normal lung parenchyma (157).

Plasma metabolomics offers the potential for developing clinically relevant liquid-biopsy solutions for cancer detection and prediction of therapy benefit. However, NSCLCs display intratumor metabolic heterogeneity in nutrient utilization, which may be challenging for the development of markers and therapies (158). The plasma metabolomics markers need large-scale validations before their clinical utility is ascertained.

Another potential source of material from body fluids in NSCLC is urine. LC-MS analysis of urine samples collected from patients with NSCLC showed increased levels of certain amino acids, including tyrosine, tryptophan, and phenylalanine (159). Modified nucleosides, regarded as indicators for the whole-body turnover of RNAs, are excreted in abnormal amounts in the urine of patients with various malignancies, including NSCLC (160).

TEPs

TEPs can sequester approximately 5500 RNA biomarkers and are considered a promising biosource for cancer detection (161,162). The mechanism for platelet education in tumors remains largely elusive. Platelet pre-mRNA in cancer patients can undergo premature splicing and translation, which results in their activation, likely enhancing the thrombo-embolic state. Platelets alter their RNA content upon cancer-associated cues, however, overall,

the RNA transcripts enriched in TEPs are ontologically associated with platelet activity and platelet vesicles. Platelet RNA profiles discriminate between patients with localized and metastatic disease and the healthy individuals with an accuracy of 84–96%. Notably, this seems possible with only minute amounts of platelet RNA (100–500 picograms) extracted from routinely used volumes of blood samples. Machine learning–based classification algorithms were found to predict the site of the primary tumor with 71% accuracy, which may potentially define the molecular subtype of NSCLC (163). In a follow-up study, the accuracy of TEP-based detection of early- and advanced-stage NSCLC was 81% and 88%, respectively, independent of patient age, smoking habits, whole-blood storage time, and various inflammatory conditions (163). However, further large-scale validation showed lower sensitivity of TEP-based assays in NSCLC (50%, 70%, 63%, and 77% for stages I, II, III, and IV, respectively), at the 99% specificity (161). However, the assay specificity dropped to an average of 78% if the controls included individuals with symptomatic inflammatory and cardiovascular diseases and benign tumors. This limits the potential application of current pan-cancer TEPs-based tests to only asymptomatic individuals. Hence, the TEP-based algorithms still require refinement.

TEPs have also been shown to sequester tumor-derived EML4–ALK fusion transcripts that displayed lowered titer upon successful crizotinib therapy in a patient with NSCLC (162). The platelet lifespan is around 7 to 10 days, and tumor-derived transcript can accumulate in the TEPs and be protected from plasma RNases. Therefore, TEP RNA analysis may enable higher sensitivity of detection and, thus, more accurate monitoring of response to treatment.

The performance of TEPs-based detection of cancer is somewhat lower than ctDNA/protein-based tests (69–98% sensitivity at 99% specificity for Cancer Seek and 80–85% sensitivity at 99% specificity for Cancer Radar) (161,163-165). The clinical usefulness of TEPs as a biomarker warrant further investigation.

Extracellular membrane vesicles (ExCeMVs)

ExCeMVs comprise a pool of small vesicles released by cells as a part of normal or pathological cell processes. These vesicles include apoptotic and necrotic bodies and exosomes (166). Exosomes, ranging from 40 to 160 nanometers, are actively released from living cells (167). ExCeMVs contain various molecules, such as lipids, proteins, and RNA, and are considered a mode of intercellular communication, contributing to a wide range of biological processes, including cancer (167,168). Alterations in exosomal cargo content can serve as diagnostic and prognostic biomarkers. As an example, a panel of three exosomal proteins (CD151, CD171, and tetraspanin 8) has been shown to be a promising diagnostic marker in NSCLC (169), whereas elevated levels of exosomal membrane–bound protein NY-ESO-1 have been associated with poor prognosis (165). Interestingly, exosomal content may also prompt metastasis formation. Amphiregulin carried by exosomes derived from NSCLC may induce EGFR pathway activation in pre-osteoclasts, which results in the expression of receptor-activator-of-nuclear-factor-kappa-B-ligand (RANKL). RANKL, in turn, increases the expression levels of several proteolytic enzymes, triggering a vicious circle in osteolytic bone metastases. Expression levels of exosomal ZEB1 are associated with significantly higher resistance to cisplatin and gemcitabine and may inform treatment decisions (170). Interestingly, exosomes from T790M-positive cells can induce resistance to gefitinib in sensitive cells via activation of the PI3K/AKT signaling pathway (171), whereas exosomal transfer of wild-type EGFR may confer osimertinib resistance (172). In turn, exosomal transfer of miR-7 may restore gefitinib sensitivity in previously resistant cells (173).

Importantly, interrogation of exosome RNA/DNA content was shown to improve the detection of T790M resistance mutations to the first and second generation of EGFR TKIs. For example, the sensitivity and specificity of the exosome-based T790M mutation detection combined with ctDNA detection were 92% and 89%, respectively, with ExoDx Lung test, compared to 58% and 80%, respectively, with the FDA-approved cfDNA test (cobas EGFR Mutation Test v2, Roche) (174). Interestingly, the exosome-based test showed an unparalleled performance for the detection of T790M resistance mutation in the case of intrathoracic disease that is difficult to detect by liquid biopsy, with sensitivity and specificity of 88% and 94% for disease stages M0/M1a and M1b, respectively. Such combined exosome/cfDNA platform was also shown to generate approximately ten-fold more copies of EGFR-activating mutations compared to cfDNA-based BEAMing analysis and a higher clinical sensitivity (175). The dual approach addressing two biological processes of the tumor, with living cells shedding the exosomes and cfDNA being released by necrotic/apoptotic cells, yields better sensitivity and may facilitate earlier detection of the developing

resistance to TKIs.

Circulating cell-free RNA (cfRNA)

Several earlier reports suggested that alterations in the plasma cfRNA can be detected in cancer patients; however, this biosource was considered less stable and thus less robust than cfDNA pool (176,177). However, a recent comprehensive study showed that samples collected in cfDNA-preserving tubes stored for up to 48 hours at room temperature allow for reliable plasma cfRNA assessment (178). This study also showed that cfRNA is specific for cancer origin and identified 15 mRNA transcripts potentially useful for detecting breast and lung cancers. Another recent study interrogated cfRNA as a potential biosource specific for multiple myeloma and liver cancer detection (179). Identified biomarkers included liver- or bone marrow-specific mRNAs related to cell-cycle processes. The levels of cfRNAs were increasing from the lowest in noncancerous states, intermediate in precancerous conditions, to highest in cancer. Further, the plasma cfRNA turned out to contain biomarkers providing the cue for cancer origin, which likely recruit from the tumor microenvironment and may reflect the healthy tissue response to the tumor. The plasma volume required for RNA extraction was 3 mL, without the extra steps of extracellular vesicles extraction.

Another interesting category of cfRNAs are cell-free microRNAs (cf-miRNAs), characterized by high stability even in harsh conditions (180). A systematic review, including early-stage NSCLC, showed a sensitivity exceeding 80% for miR-223, miR-20a, miR-448, and miR-145 and a specificity exceeding 90% for miR-628-3p, miR-29c, miR-210, and miR-1244 (181). In a cohort study including more than 3,000 patients, a panel of cf-miRNAs identified patients with lung cancer with 91.4% accuracy, 82.8% sensitivity, and 93.5% specificity (182). The prognostic value of cf-miRNAs was assessed in a study including 192 NSCLC patients (99 adenocarcinomas and 83 squamous cell carcinomas) (183). Of the 68 miRNAs analyzed, the most predictive for the outcome was cf-miR-126, with low expression predicting poor prognosis. This finding was confirmed in the meta-analysis including 1,012 patients (184).

Currently, the clinical usefulness of cfRNA for cancer detection requires further validation, particularly in screening for early-stage disease in the background of concomitant disorders, e.g., precancerous states or COPD. The targeted cfRNA appears to be a promising analyte to enhance the sensitivity of cfDNA-based cancer detection, particularly in low-shedding tumors. Lastly, a multianalyte test comprising both cfRNA and cfDNA may improve tissue of origin determination, which is vital to pan-cancer screening approaches.

Conclusions

Liquid biopsy has revolutionized the oncology field after overcoming several of the limitations of traditional tissue biopsy techniques. This innovative non-invasive approach has the unquestionable potential to optimize NSCLC management. Currently, it constitutes a valuable diagnostic tool for identifying druggable molecular alterations. New applications may include early detection, real-time monitoring, and the evaluation of spatial and temporal NSCLC heterogeneity.

We discussed the two most commonly used approaches, ctDNA/cfDNA and CTCs, and emerging liquid biopsy techniques, such as plasma DNA methylation, plasma cfRNA and metabolites, extracellular vesicles, and platelets. We also highlighted the limitations hampering clinical applications of liquid biopsies. We summarized the most up-to-date results of ongoing clinical trials and presented studies whose results may shortly impact clinical practice. The greatest hopes lie in MRD assessment, which may guide adjuvant therapies and allow early relapse detection and faster initiation of salvage treatments. However, the clinical application of liquid biopsies is still limited due to their suboptimal specificity and sensitivity, lack of standardization, and relatively high costs. Addressing these issues may allow further integration of liquid biopsies in the routine clinical setting, thus making a profound and permanent change in NSCLC management.

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Footnote

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