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Review

Liquid Biopsy and Immuno-Oncology for Advanced Nonsmall Cell Lung Cancer

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BACKGROUND: In the last decade, immune checkpoint inhibitors have revolutionized the treatment of metastatic nonsmall cell lung cancer without oncogenic addiction. Currently, programmed death ligand 1 (PD-L1) status, assessed in tissue biopsy samples, is the only test for guiding the prescription of these therapies in clinical practice. However, obtaining tumor tissue from patients with lung cancer is not always feasible and PD-L1 positivity is not a guarantee of immunotherapy efficacy. In this context, liquid biopsy, represented by several circulating biomarkers that reflect the tumor characteristics, is emerging as an interesting alternative approach.

CONTENT: We describe the main blood biomarkers evaluated in patients with metastatic nonsmall cell lung cancer before/during immune checkpoint inhibitor treatment, with a focus on circulating cell-free DNA, circulating tumor DNA (ctDNA), blood tumor mutational burden, and circulating tumor cells (CTCs).

SUMMARY: Monitoring of ctDNA and CTCs during immunotherapy may be a promising tool to help clinicians in therapeutic decision-making.

Introduction

Recently, the prognosis of advanced nonsmall cell lung cancer (NSCLC) has been considerably improved by tyrosine kinase inhibitors (TKIs) and immune checkpoint inhibitors (ICIs).

In the absence of epidermal growth factor receptor (*EGFR*) alterations or anaplastic lymphoma kinase rearrangements, antibodies against programmed death ligand 1 (PD-L1) and programmed death 1 (PD-1) are now widely used in second- (1) and first-line settings (2). In first-line settings, pembrolizumab can be used alone if PD-L1 is expressed on at least 50% of tumor cells, or in combination with chemotherapy in the other cases (3). In second-line settings, pembrolizumab, nivolumab, or atezolizumab can be considered (1, 4, 5).

A comprehensive understanding of the predictive factors of the response to these agents is required for optimal patient selection. Indeed, the prognosis of patients with advanced NSCLC remains poor, highlighting the need for accurate treatment choice. Moreover, ICIs can induce severe immune-related adverse events, such as heart, lung, or liver toxicity (6). New targeted therapies are emerging for the management of metastatic NSCLC. For instance, sotorasib, a KRAS inhibitor, has recently been approved for previously treated patients with NSCLC harboring the *KRAS* p.G12C mutation (7), and other TKIs are under investigation in this setting (8). The optimal place of anti-PD-(L)1 antibodies among these innovative molecules needs to be determined. In clinical practice, PD-L1 tissue expression is the only validated biomarker to guide the prescription of immunotherapy. However, accumulating evidence shows that PD-L1 expression analysis by immunohistochemistry alone is not always reliable enough to predict the response to ICIs, notably because of its spatial heterogeneity (9). The concept of circulating cancer biomarkers, referred to as "liquid biopsy," which can be assessed in various biological fluids, such as blood, urine, saliva, or cerebrospinal fluid (10), has been introduced in the last decade to overcome this problem. It has been proposed that circulating biomarkers represent more accurately the whole tumor characteristics and heterogeneity. Furthermore, since liquid biopsy is less invasive than tissue biopsy, samples can be repeatedly collected over time to provide real-time information on the tumor behavior.

In this review, we discuss the results of studies on blood biomarkers as predictive markers of the response to ICIs in patients with advanced NSCLC, with a focus on circulating cell-free DNA (cfDNA), circulating

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tumor DNA (ctDNA), blood tumor mutational burden, and circulating tumor cells (CTCs).

We selected studies in the PubMed database using the following terms (as text and as MeSH terms): "non-small cell lung cancer" plus "immunotherapy" or "immune checkpoint inhibitors" or "anti-PD-1" or "anti-PD-L1" plus "circulating cell-free DNA" or "circulating tumor DNA" or "blood tumor mutational burden," or "circulating tumor cells" or "PD-L1 positive circulating tumor cells."

Circulating Tumor DNA and Circulating-free DNA

In NSCLC, ctDNA is probably the most studied liquid biopsy-based biomarker. Different sensitive detection methods have been developed because the fraction of tumor-specific cfDNA varies between 0.1% and >90% in patients with cancer (11). In clinical practice, ctDNA is widely used in patients with NSCLC to detect *EGFR* mutations when tumor tissue is not available, at diagnosis or during tumor progression, to determine the mechanisms of resistance to TKIs (12). Moreover, different studies have shown that dynamic changes in plasma ctDNA levels correlate with EGFR-TKI therapeutic efficacy (13, 14). In patients with NSCLC receiving immunotherapy, ctDNA clinical relevance has not been formally demonstrated. However, many studies have evaluated ctDNA/cfDNA as a biomarker of response to ICIs, alone or in combination with other treatments (particularly chemotherapy) (Table 1). In this context, ctDNA analysis is based on the identification of the somatic mutation with the highest allele frequency, assessed by next-generation sequencing (NGS). Then, ctDNA level is expressed by its concentration or the fraction of mutant alleles (maximum somatic allele frequency or MSAF). Other authors use the mean variant allele frequency (VAF) that corresponds to the mean allele frequency of all detected somatic variants.

Some studies have found that a low ctDNA level at baseline (i.e., before ICI initiation) is associated with improved overall survival (OS) (15, 20, 24, 29, 31). However, the most relevant cutoff to define high/low ctDNA level needs to be determined.

Results are more consistent when ctDNA kinetics are measured during treatment. Often, ctDNA decrease during immunotherapy has been associated with improved response to treatment, durable clinical benefits (i.e., >6 months), and longer progression-free survival (PFS) and OS (15–19, 21, 27, 28, 30, 31, 33). Importantly, 2 studies showed that early ctDNA changes (i.e., 2–3 weeks after immunotherapy initiation) can predict the response/resistance to treatment (16, 27). Moreover, the molecular response (i.e., change in ctDNA levels) is observed generally earlier than the radiologic response (17, 21, 27), highlighting the potential of ctDNA as an early response biomarker.

Changes in ctDNA during ICI-based therapy could be a promising tool to improve treatment decision-making (Fig. 1). However, the gene number and mutations tested and the allele frequency cutoff used for their detection are hugely variable among NGS-based studies. Sometimes, only mutations found both in the tissue biopsy and ctDNA were considered (16, 21), which reduces the number of patients eligible for this molecular monitoring.

Only a few studies have evaluated cfDNA, reporting that a low baseline cfDNA level is associated with better clinical outcomes; however, longitudinal monitoring has not always been conclusive (22, 29, 32). Mondelo-Macia et al. reported that a decrease in cfDNA levels at 12 weeks was associated with improved PFS in 50 NSCLC patients treated with pembrolizumab, alone or in combination with chemotherapy, in the first-line setting (32). However, Park et al. did not find any clinical impact of cfDNA changes in a smaller cohort of pretreated NSCLC patients, receiving pembrolizumab or atezolizumab, using a different detection method (29).

Blood Tumor Mutational Burden

The tumor mutational burden (TMB) is defined as the number of somatic mutations per megabase (mut/Mb) of sequenced genome. The presence of these variants may lead to the formation of neoantigens that are recognized by the immune system as nonself, resulting in the antitumor immune response activation. This suggests that patients with NSCLC and high TMB could benefit from immunotherapy. Several findings support the use of TMB assessed in tissue biopsy samples (tTMB) as a biomarker of ICI efficacy in advanced NSCLC (34, 35). However, recent exploratory analyses in the framework of phase III studies suggest that tTMB has limited clinical utility. In the Checkmate-227 trial, OS benefit was comparable with nivolumab plus ipilimumab and with chemotherapy, regardless of the (high or low) tTMB (36). A pooled analysis of the Keynote 021, 189, and 407 studies gave similar results concerning the pembrolizumab plus chemotherapy combination (37).

Since TMB cannot usually be assessed due to the lack of tumor tissue or the low percentage of tumor cells in the biopsy, several groups investigated whether it could be measured in cfDNA. In 2017, Fabrizio et al. developed and analytically validated a blood-based assay to determine blood tumor mutation burden (bTMB) in patients with NSCLC (38). The assay limit of detection was defined as 1% of tumor content in at least 20 ng of

Table 1. Studies assessing cf/ctDNA level as predictive factor of response to ICIs in patients with advanced NSCLC.

Patient population	Study type	Treatment	Ct/cfDNA analysis methods	Clinical outcomes
15 patients with metastatic NSCLC (+ uveal melanoma or colorectal cancer) Cabel et al. (15)	Prospective	Nivolumab or pembrolizumab, 2nd line or more	NGS (39 genes), ddPCR, or Bi-PAP	-**Baseline undetectable ctDNA associated with improved OS** (univariate analysis) -**Complete ctDNA clearance at 8 weeks associated with OR and undetectable ctDNA at 8 weeks associated with improved PFS and OS** (univariate analysis)
14 patients with metastatic NSCLC Iijima et al. (16)	Retrospective	Nivolumab, 2nd line	NGS (53 genes)	-Baseline ctDNA level not associated with response at the 2nd radiologic evaluation -Decrease in MSAF at 2 weeks in all responders at the 2nd evaluation and increase in MSAF at 2 weeks in all nonresponders at the 2nd evaluation
28 patients with metastatic NSCLC Goldberg et al. (17)	Prospective	Anti-PD-1 or anti-PD-L1 ± anti-CTLA4 antibodies, treatment line unknown	NGS (24 genes)	**ctDNA response at any time** (drop in ctDNA level <50% of baseline) **correlated with OR as best response to treatment, DCB, improved PFS and OS** (univariate analysis)
15 patients with stage III and IV NSCLC Giroux Leprieur et al. (18)	Prospective	Nivolumab, 2nd line (87%) or more	NGS (22 genes)	-Baseline ctDNA level associated neither with OR at 8 weeks nor with DCB -**ctDNA concentration <0.006 ng mL^{-1} at 8 weeks associated with improved PFS and OS** (univariate analysis), and **no increase in ctDNA level at 8 weeks associated with OR, DCB, improved PFS and OS** (univariate analysis)
28 (discovery cohort) and 72 (validating cohort) patients with stage III and IV NSCLC Raja et al. (19)	Retrospective analysis of 2 randomized trials	Durvalumab, 1st and 2nd line or more	NGS (73 genes)	**Decrease in mean VAF at 6 weeks associated with OR as best response to treatment, DCB, improved PFS and OS** (multivariate analysis)

Continued

Table 1. (continued)

Patient population	Study type	Treatment	Ct/cfDNA analysis methods	Clinical outcomes
20 (discovery cohort) and 12 (validating cohort) patients with stage III and IV NSCLC Chae et al. (20)	Retrospective	Anti-PD-1 or anti-PD-L1 antibodies, 1st and 2nd line or more	NGS (73 genes)	**High baseline MAF** (above the median) **associated with poor OS** (multivariate analysis)
24 patients with metastatic NSCLC Anagnostou et al. (21)	Retrospective	Anti-PD-1 ± anti-CTLA4, anti-LAG3 antibodies or chemotherapy, treatment line unknown	NGS (58 genes)	**Complete ctDNA clearance at any time associated with improved PFS and OS** (univariate analysis)
89 patients with advanced NSCLC Alama et al. (22)	Prospective	Nivolumab, 2nd line or more	qPCR using hTERT gene copy number	-Baseline cfDNA level not associated with the best response to treatment **-Low baseline cfDNA level** (concentration ≤836.5 ng/3 mL) **associated with improved OS** (multivariate analysis)
12 patients with stage III and IV NSCLC Li et al. (23)	Pilot study	Pembrolizumab alone or in combination with chemotherapy, 1st or 2nd line	NGS (329 genes)	Higher baseline MSAF in the SD/PD group than in the PR group
64 patients with stage III and IV NSCLC Wang et al. (24)	Retrospective	Anti-PD-1 or anti-PD-L1 antibodies, 1st and 2nd line or more	NGS (150 genes)	**High baseline MSAF** (top 25%) **associated with worse OS** (multivariate analysis)
31 patients with metastatic NSCLC with PFS >12 months Hellmann et al. (25)	Retrospective	Anti-PD-1 or anti-PD-L1 antibodies, alone or in combination with anti-CTLA4, anti-LAG3 antibodies, bevacizumab or erlotinib, 1st	NGS (number of genes not available)	**Undetectable ctDNA during treatment associated with longer event-free survival since blood collection** (univariate analysis)

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Table 1. (continued)

Patient population	Study type	Treatment	Ct/cfDNA analysis methods	Clinical outcomes
		(72%) and 2nd line or more		
22 patients with stage III and IV NSCLC Chen et al. (26)	Pilot study	Camrelizumab + apatinib, 2nd line (64%) or more	NGS (605 genes)	**Higher baseline ctDNA level associated with worse PFS** (multivariate analysis), but no difference when dichotomized in "ctDNA negative" and "ctDNA positive"
45 patients with advanced NSCLC Ricciuti et al. (27)	Retrospective	Pembrolizumab alone or in combination with chemotherapy, 1st line	NGS (36 genes)	**Decrease in MSAF at 3 weeks associated with OR as best response to treatment, PFS and OS** (multivariate analysis)
45 patients with metastatic NSCLC Thompson et al. (28)	Prospective	Pembrolizumab alone or in combination with chemotherapy, 1st or 2nd line	NGS (74 genes)	**Decrease in mean VAF >50% at 9 weeks associated with OR, DCB, PFS, and OS** (univariate analysis)
24 patients with stage III and IV NSCLC Park et al. (29)	Prospective	Pembrolizumab or atezolizumab, 2nd line or more (97%)	Fluorometric-based method	-**Low baseline cfDNA level** (concentration ≤5.8 ng mL^{-1}) **associated with improved OS** (univariate analysis) -Change in cfDNA level during treatment not associated with clinical outcomes
94 patients with advanced NSCLC Zou et al. (30)	Retrospective analysis of a phase III trial	Atezolizumab versus chemotherapy, 2nd line	NGS (197 genes)	-**Low MMPM at 6 weeks associated with improved OS in both arms** (univariate analysis) -**Decrease in maximum AF >50% at 6 weeks associated with OS in both arms** (univariate analysis)
100 patients with advanced lung adenocarcinoma van der Leest et al. (31)	Retrospective	Anti-PD-1 or anti-PD-L1 antibodies, 1st (25%) and 2nd line or more	ddPCR	-**Low baseline ctDNA level** (below the median) **associated with improved PFS and OS** (univariate analysis) -**ctDNA decrease at 4–6 weeks associated with DCB, improved PFS and OS** (univariate analysis)
50 patients with advanced NSCLC	Prospective	Pembrolizumab alone or in combination with	qPCR using hTERT gene copy number	-**Low baseline cfDNA level** (≤2132.39 GE/mL for PFS and ≤2075.59 GE/mL for OS)

Continued

Table 1. (continued)

Patient population	Study type	Treatment	Ct/cfDNA analysis methods	Clinical outcomes
Mondelo-Macía et al. (32)		chemotherapy, 1st line		**associated with improved PFS and OS** (univariate analysis) -**Low cfDNA levels at baseline and at 12 weeks or cfDNA decrease at 12 weeks associated with improved PFS** (univariate analysis)
134 patients with stage III and IV squamous NSCLC Ren et al. (33)	Exploratory analysis of a phase III trial	Camrelizumab + chemotherapy, 1st line	NGS (number of genes not available)	-Baseline ctDNA level not associated with response to treatment -**Low mean VAF** (below the median) **after 2 cycles associated with OR,** and **undetectable ctDNA at baseline and after 2 cycles or ctDNA decrease after 2 cycles associated with improved PFS and OS** (univariate analysis)

Bold font indicate statistically significant associations.
Abbreviations: AF, allele frequency; Bi-PAP, bidirectional pyrophosphorolysis activated polymerization; DCB, durable clinical benefit; ddPCR, droplet-digital PCR; GE/mL, genome equivalents per milliliter; MAF, mutant allele frequency; MMPM, mutant molecules per milliliter; MSAF, maximum somatic allele frequency; NGS, next-generation sequencing; OS, overall survival; OR, objective response; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; VAF, variant allele frequency.

cfDNA (≥1% of MSAF). Table 2 lists published studies that evaluated bTMB clinical utility in patients with advanced NSCLC treated with ICIs. One of the first published studies was a retrospective analysis of 2 large randomized trials on atezolizumab as second or further line treatment (39). Analyses performed in blood samples from patients in the POPLAR study demonstrated that bTMB ≥16 mutations per 1.1 megabase is a clinically meaningful and technically robust cutoff point in patients with NSCLC. These results were validated using blood samples from the OAK study. Although bTMB and tTMB were positively correlated (Spearman rank correlation: 0.64; 95% confidence interval (CI): 0.56–0.71), a lower proportion of tumor-derived plasma DNA and a longer interval between tissue and plasma sampling were associated with a higher discordance rate. Numerous other studies have found a high bTMB to be predictive of longer PFS in patients with NSCLC treated with ICIs (23, 39–43), and one study showed an association with OS (44). However, 2 studies failed to confirm the positive impact of high bTMB in their validation cohorts (20, 45). Moreover, Wang et al. did not find any correlation between bTMB and clinical outcomes using a threshold of 6 mut/Mb (24). These results are supported by 2 larger prospective trials. The phase II B-F1RST trial investigated PFS according to the bTMB as primary endpoint in 119 patients with advanced NSCLC who received first-line atezolizumab. PFS and OS were longer in patients with bTMB ≥14.5 mut/Mb vs <14.5 mut/Mb, but the difference was not significant (PFS: 5.0 vs 3.5 months, HR 0.80, 90% CI: 0.54–1.18 and OS: 23.9 vs 13.4 months, HR 0.66, 90% CI: 0.40–1.10) (46). Similarly, the phase III NEPTUNE trial (NCT02542293) that compared first-line durvalumab and tremelimumab vs platinum-based chemotherapy did not meet the primary endpoint of OS improvement in patients with high bTMB (≥20 mut/Mb) (47). Four phase III trials explored bTMB predictive value in patients with NSCLC who received immunotherapy in combination with chemotherapy. Wang et al. reported a PFS improvement with the tislelizumab plus chemotherapy combination, regardless of the bTMB (48), but with significant results only in the high bTMB group. In the other 3 trials, the combination treatment benefit was not influenced by bTMB (49–51).

All this data shows that bTMB is inconsistently associated with clinical outcomes in patients with advanced NSCLC receiving ICIs. This could be partly explained by the lack of standardization in detection methods, as for ctDNA level. Indeed, the panel of sequenced genes, the mutations analyzed, the allele frequency cutoff used for their selection, and the mut/Mb threshold to define high bTMB differ significantly

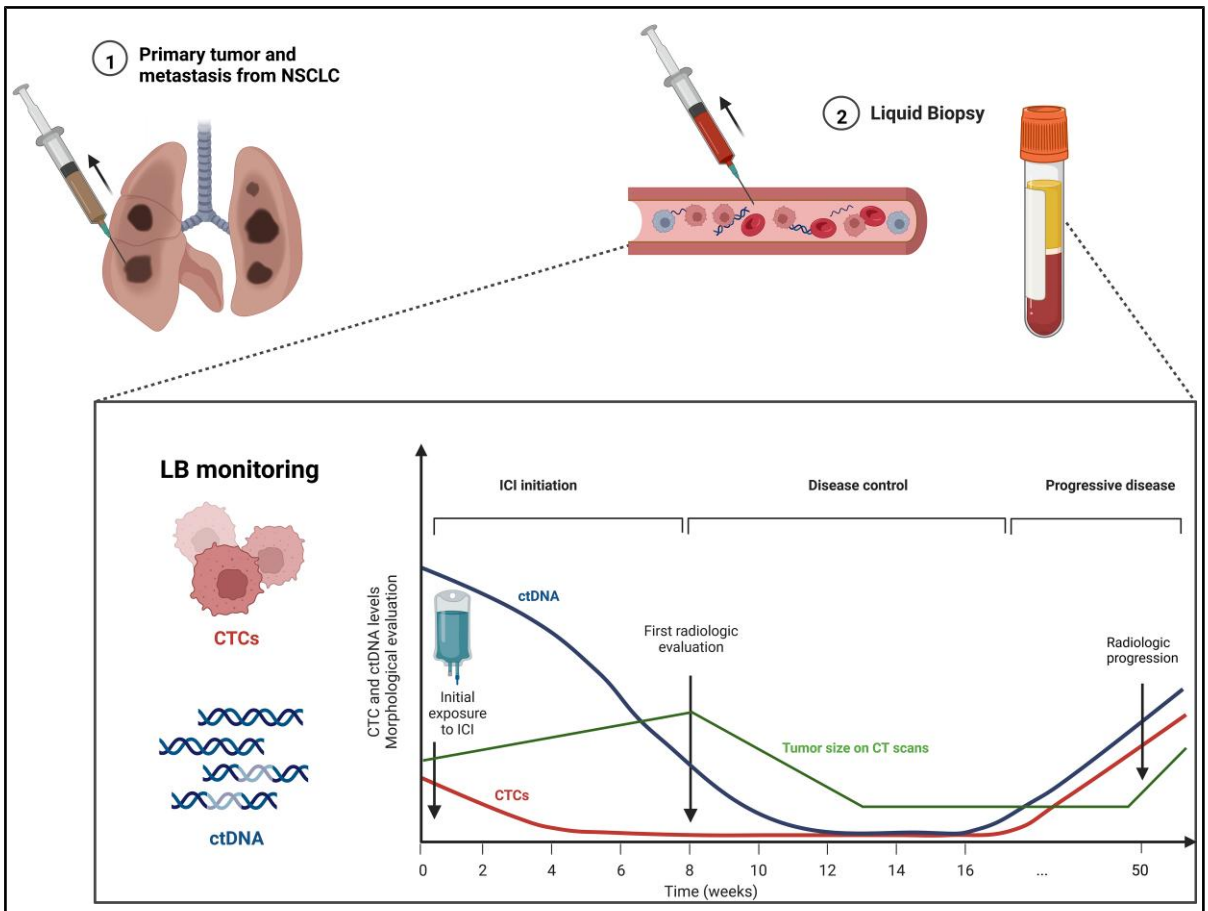

Fig. 1. Compared to tissue biopsy (1), liquid biopsy (LB) (2) can be used to capture both spatial and temporal tumor heterogeneity and to monitor the subclonal evolution of the disease during treatment with ICIs. CTC and ctDNA analysis can also be used to distinguish between pseudoprogression and true progression, and during the phase of disease control (minimal residual disease) to anticipate new progressions. Created with BioRender.com. Abbreviations: CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; ICIs, immune checkpoint inhibitors; LB, liquid biopsy.

among studies. Nabet et al. suggested that the ctDNA amount, which is positively correlated with the bTMB level and is associated with a negative prognostic value in NSCLC, could be responsible for the bTMB failure to predict OS in patients with NSCLC receiving ICIs (52). In their study, "normalized bTMB" (relative to ctDNA concentration) predicted PFS better than bTMB. Similarly, Wang et al. used an allele frequency-adjusted bTMB (24) and demonstrated that only high "low-allele frequency bTMB" (i.e., bTMB >12 mut/Mb when only mutations with an allele frequency <5% were considered) was predictive of longer OS in patients with NSCLC treated with anti-PD-(L) 1 agents.

Finally, dynamic bTMB changes also could be of interest. In the phase III study that compared camrelizumab plus chemotherapy vs chemotherapy alone, a decrease in bTMB after 2 cycles of the combination treatment was associated with longer PFS (50). These results need to be validated in independent studies.

Circulating Tumor Cells

CTCs can provide complete information about the tumor characteristics because they can be used for genomic, proteomic, transcriptomic, and secretomic analyses (53). However, their detection and complete isolation require very sensitive technologies due to their very low numbers in the bloodstream (approximately one CTC per 10^6–10^7 leukocytes) and their phenotypic changes over time (10). CTC detection includes a first

Table 2. Studies assessing baseline bTMB as predictive factor of response to ICIs in patients with advanced NSCLC.

Patient population	Study type	Treatment	bTMB analysis methods and cutoffs	Clinical outcomes
287 (discovery cohort) and 850 (validating cohort) patients with advanced NSCLC Gandara et al. (39)	Retrospective analysis of 2 large randomized trials	Atezolizumab versus chemotherapy, 2nd line or more	NGS (Foundation One, panel of 394 genes), 16 mutations per 1.1 megabases, equivalent to ~14.5 mut/Mb	**High bTMB (≥16 mut/ 1.1 Mb) associated with improved PFS** but not OS, **for atezolizumab versus chemotherapy** (univariate analysis). No difference in the low bTMB group
50 patients with stage III and IV NSCLC Wang et al. (41)	Retrospective	Anti-PD-1 or anti-PD-L1 antibodies, 1st and 2nd line or more	NGS (NCC-GP150, panel of 150 genes), 6 mut/Mb	**High bTMB (≥6 mut/Mb) associated with improved ORR and PFS** (multivariate analysis)
20 (discovery cohort) and 12 (validating cohort) patients with stage III and IV NSCLC Chae et al. (20)	Retrospective	Anti-PD-1 or anti-PD-L1 antibodies, 1st and 2nd line or more	NGS (Guardant360, panel of 73 genes), median bTMB (14.5 mut/Mb)	-**High bTMB (≥14.5 mut/ Mb) associated with worse PFS and OS in the discovery cohort** (univariate analysis). -bTMB not associated with clinical outcomes in the validation cohort
12 patients with stage III and IV NSCLC Li et al. (23)	Pilot study	Pembrolizumab alone or in combination with chemotherapy, 1st or 2nd line	NGS (Qiyuan, panel of 329 genes), median bTMB (21 mut/Mb)	**High bTMB (>21 mut/Mb) associated with improved PFS** (univariate analysis)
64 patients with stage III and IV NSCLC Wang et al. (24)	Retrospective	Anti-PD-1 or anti-PD-L1 antibodies, 1st and 2nd line or more	NGS (NCC-GP150, panel of 150 genes), 6 mut/Mb	bTMB not associated with clinical outcomes
1118 patients with metastatic NSCLC Rizvi et al. (44)	Pre-specified exploratory analysis of a phase III trial	Durvalumab versus durvalumab + tremelimumab versus chemotherapy, 1st line	NGS (Guardant OMNI, panel of 500 genes), 20 mut/Mb	**High bTMB (≥20 mut/Mb) associated with improved ORR, PFS, and OS for durvalumab + tremelimumab versus chemotherapy** (univariate analysis). No improvement in the low bTMB group
66 patients with metastatic NSCLC	Prospective	Pembrolizumab alone or in	NGS (Guardant OMNI,	-**Median bTMB significantly higher for**

Continued

Table 2. (continued)

Patient population	Study type	Treatment	bTMB analysis methods and cutoffs	Clinical outcomes
Aggarwal et al. (40)		combination with chemotherapy, 1st line	panel of 500 genes), 16 mut/Mb	**patients with OR at 9 weeks and OR/SD at 6 months** (multivariate analysis) -**High bTMB (≥16 mut/Mb) associated with improved PFS** but not OS (multivariate analysis)
22 patients with stage III and IV NSCLC Chen et al. (26)	Pilot study	Camrelizumab + apatinib, 2nd line (64%) or more	NGS (HaploX, panel of 605 genes), 66.7 percentile bTMB value (6.96 mut/Mb)	bTMB not associated with clinical outcomes
554 patients with metastatic NSCLC with PD-L1 ≥ 1% (tumor cells and/or immune cells) Herbst et al. (42)	Pre-specified subgroup analysis of a phase III trial	Atezolizumab versus chemotherapy, 1st line	NGS (Foundation One, panel of 394 genes), 16 mutations per 1.1 megabases, equivalent to ~14.5 mut/Mb	**High bTMB (≥16 mut/1.1 Mb) associated with improved PFS** but not OS **for atezolizumab versus chemotherapy** (univariate analysis). No improvement in the low bTMB group
13 patients with advanced NSCLC Ma et al. (43)	Retrospective	Pembrolizumab or nivolumab, 2nd line	NGS (xGen Lockdown Probes, panel of 547 genes), 6 mut/Mb	**High bTMB (≥6 mut/Mb) associated with improved ORR and PFS,** but not OS (univariate analysis)
42 (exploratory cohort) and 14 (validating cohort) patients with stage III and IV NSCLC Chen et al. (45)	Prospective	Anti-PD-1 or anti-PD-L1 antibodies, 1st (5%) and 2nd line or more	NGS (OncoScreen, panel of 520 genes), 11 mut/Mb	-**High bTMB (≥11 mut/Mb) associated with improved PFS in the exploratory cohort** (univariate analysis) -bTMB not associated with clinical outcomes in the validating cohort
389 patients with stage III and IV NSCLC (squamous cell carcinoma only) Jiang et al. (50)	Pre-specified exploratory analysis of a phase III trial	Camrelizumab + chemotherapy versus chemotherapy alone, 1st line	NGS (HyperCap Target Enrichment Kit, panel of 543 genes), 75%	bTMB not associated with clinical outcomes

Abbreviations: BEP, biomarker evaluable population; ITT, intention to treat population; mut/Mb, mutations per megabase; NGS, next-generation sequencing; OR, objective response; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; SD, stable disease.

Table 3. Studies assessing CTCs as predictive factor of response to ICIs in patients with advanced NSCLC.

Patient population	Study type	Treatment	CTC detection methods	Clinical outcomes
17 patients with metastatic NSCLC Dhar et al. (57)	Pilot study	Nivolumab, pembrolizumab, avelumab, nivolumab + ipilimumab, treatment line unknown	Vortex HT Chip (inertial forces)	Baseline CTC count not associated with PFS
96 patients with metastatic NSCLC Guibert et al. (58)	Prospective	Nivolumab, 2nd line	ISET (size)	**-High baseline CTC count** (>30/7.5 mL of blood) **associated with worse PFS and OS** (univariate analysis) -Median CTC number higher at disease progression
11 patients with stage III and IV NSCLC Janning et al. (59)	Prospective	Nivolumab, pembrolizumab, atezolizumab, 1st, 2nd, and 3rd line	Parsortix (size, deformability)	**CTC decrease or stability at 3–5 weeks in 89% of nonprogressive patients versus increase, at progression time, in all progressive patients**
104 patients with stage III and IV NSCLC Tamminga et al. (60)	Prospective	Nivolumab (85%), pembrolizumab, atezolizumab, nivolumab + ipilimumab, 2nd line or more (96%)	CellSearch® (EpCAM)	**-CTC presence at baseline or at 4 weeks** not associated with early response (4–6 weeks), but correlated **with lower durable response** (>6 months) **-CTC presence at baseline associated with worse PFS and OS** (multivariate analysis) **-CTC increase at 4 weeks associated with worse PFS and OS** (multivariate analysis)
89 patients with advanced NSCLC Alama et al. (22)	Prospective	Nivolumab, 2nd line or more	ScreenCell (size)	-Baseline CTC count not associated with best response to treatment **-High baseline CTC count** (>2/3 mL of blood) **associated with worse OS** (multivariate analysis)
35 patients with metastatic or relapsed NSCLC Castello et al. (61)	Prospective	Nivolumab (57%), pembrolizumab, atezolizumab, nivolumab + ipilimumab, 1st (26%), and 2nd line or more	ISET	-Baseline CTC count not associated with clinical outcomes **-CTC increase at 8 weeks associated with metabolic progressive disease,** and **high CTC count** (>11/10 mL of blood) **at 8 weeks associated with worse PFS and OS** (multivariate analysis)

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Table 3. (continued)

Patient population	Study type	Treatment	CTC detection methods	Clinical outcomes
15 patients with advanced NSCLC Papadaki et al. (62)	Prospective	Anti-PD-1 antibodies, 2nd and 3rd line	ISET and Parsortix	-**CTC presence at baseline** (detected by Parsortix) **associated with progressive disease as best response to treatment** -**CTC presence at baseline** (detected by any method) **associated with worse PFS,** but not with OS (univariate analysis)
50 patients with advanced NSCLC Mondelo-Macía et al. (32)	Prospective	Pembrolizumab alone or in combination with chemotherapy, 1st line	CellSearch® and Parsortix	-**Higher CTC count at baseline** (detected by CellSearch) **associated with subsequent progressive disease** -**CTC presence at baseline** (detected by CellSearch) **associated with worse PFS and OS** (multivariate analysis)
83 patients with stage III and IV NSCLC Park et al. (29)	Prospective	Pembrolizumab or atezolizumab, 2nd line or more (97%)	CD-PRIME system (size)	- Baseline CTC count not associated with clinical outcomes -**CTC increase at 3 weeks associated with worse PFS and OS** (multivariate analysis)
44 patients with advanced NSCLC Ikeda et al. (63)	Prospective	Nivolumab, 2nd line or more (99%)	MCA system (size)	-**CTC increase at 8 weeks associated with nonprogressive disease**
39 patients with advanced NSCLC Dall'Olio et al. (64)	Prospective	Pembrolizumab, nivolumab or atezolizumab, 2nd line or more	CellSearch®	-**CTC presence at baseline associated with worse PFS and OS** (multivariate analysis)

Abbreviations: OS, overall survival; PFS, progression-free survival.

enrichment step based on their biological (e.g., expression of surface proteins/receptors) or physical (e.g., size, deformability, density, and electric charges) properties before the detection and characterization phase (10).

In 2015, we described the detection of PD-L1-positive (PD-L1$^{(+)}$) CTCs (54). Their clinical relevance was then shown in patients with breast cancer (55) and NSCLC (56) receiving chemotherapy (PFS and OS). In the context of liquid biopsy in immuno-oncology, several groups investigated CTCs and PD-L1$^{(+)}$ CTCs as predictive biomarkers of immunotherapy efficacy in advanced NSCLC (Tables 3 and 4, respectively).

CTC presence (or count) at baseline seems to be associated with the durable response rather than the early response to ICIs (32, 60, 61). In terms of survival, these results translated into a negative impact of baseline CTC presence (or count) on PFS and OS in most studies with large sample size (22, 32, 58, 60). Concerning

Table 4. Studies assessing PD-L1$^{(+)}$ CTCs as predictive factor of the response to ICIs in patients with advanced NSCLC.

Patient population	Study type	Treatment	CTC detection methods	Antibody against PD-L1, definition of CTC PD-L1 positivity	Clinical outcomes
17 patients with metastatic NSCLC Dhar et al. (57)	Pilot study	Nivolumab, pembrolizumab, avelumab, nivolumab + ipilimumab; treatment line unknown	Vortex HT Chip (inertial forces)	4059 (ProSci Inc), PD-L1 expressed on ≥1 CTC	Baseline PD-L1$^{(+)}$ CTC count not associated with PFS
96 patients with metastatic NSCLC Guibert et al. (58)	Prospective	Nivolumab, 2nd line	ISET (size, deformability)	D8T4X (Cell Signaling), PD-L1 expressed on ≥1% of CTCs	Baseline PD-L1$^{(+)}$ CTC presence not associated with PFS or OS. But **patients with PD-L1$^{(+)}$ CTCs more frequently nonresponders (PFS < 6 months).** At progression, PD-L1$^{(+)}$ CTCs in 23/24 assessable patients
11 patients with stage III and IV NSCLC Janning et al. (59)	Prospective	Nivolumab, pembrolizumab, atezolizumab, 1st, 2nd, and 3rd line	Parsortix	D8T4X (Cell Signaling), PD-L1 expressed on ≥1 CTC	**PD-L1$^{(+)}$ CTC decrease or stability at 3–5 weeks in all nonprogressive patients versus increase, at the time of progression, in all progressive patients**
16 patients with stage III and IV NSCLC Zhang et al. (65)	Prospective	Nivolumab, 2nd line (87%)	SE-iFISH (subtraction enrichment)	29E.2A3 (Dana-Farber Cancer Institute), PD-L1 expressed on ≥1 CTC	Baseline PD-L1$^{(+)}$ CTC presence not associated with clinical outcomes
15 patients with advanced NSCLC Papadaki et al. (62)	Prospective	Anti-PD-1 antibody, 2nd and 3rd line	ISET and Parsortix (size, deformability)	E1L3N (Cell Signaling), PD-L1 expressed on ≥1 CTC	Baseline PD-L1$^{(+)}$ CTC presence not associated with clinical outcomes
50 patients with advanced NSCLC	Prospective	Pembrolizumab alone or in combination with chemotherapy, 1st line	CellSearch® and Parsortix	Antihuman B7-H1/ PD-L1 (R&D Systems), PD-L1	Baseline PD-L1$^{(+)}$ CTC presence not associated with clinical outcomes

Continued

Table 4. (continued)

Patient population	Study type	Treatment	CTC detection methods	Antibody against PD-L1, definition of CTC PD-L1 positivity	Clinical outcomes
Mondelo-Macía et al. (32)				expressed on ≥1 CTC	
44 patients with advanced NSCLC Ikeda et al. (63)	Prospective	Nivolumab, 2nd line or more (99%)	MCA system (size)	28–8 (Abcam), PD-L1 expressed on ≥1 CTC	A majority of increase in the PD-L1$^{(+)}$ CTC count and the PD-L1 positivity rate at progression time. **PD-L1 positivity rate ≥7.7% at 8 weeks associated with improved PFS** but not with OS (univariate analysis)
39 patients with advanced NSCLC Dall'Olio et al. (64)	Prospective	Pembrolizumab, nivolumab or atezolizumab, 2nd line or more	CellSearch®	MIH3 (BioLegend), PD-L1 expressed on ≥1 CTC	**Baseline PD-L1$^{(+)}$ CTC presence associated with worse PFS and OS**, compared with CTC absence (multivariate analysis)

Abbreviations: OS, overall survival; PFS, progression-free survival.

CTC dynamics during treatment, 3 studies reported an increase in CTC count at tumor progression detection (58, 59, 61). Two studies showed that an early increase in CTC number, at week 3–4 after treatment initiation, negatively affects PFS and OS (29, 60). Only Ikeda et al. found a CTC increase in the nonprogressive group (absence of disease progression at week 8 of treatment) compared with the progressive group (disease progression between week 4 and week 8) (63). The authors suggested that this unexpected result was related to tumor tissue disruption during treatment. However, since they used an original microcavity array system for CTC enrichment from whole blood, they might have missed small-size CTCs. Indeed, this technology is based on size and deformability differences between tumor and blood cells (66) and has not yet been approved by the US Food and Drug Administration (FDA). Currently, the CellSearch system is the only FDA-cleared CTC detection method because of its standardization and reproducibility (10).

The predictive value of PD-L1$^{(+)}$ CTC on the response to immunotherapy was described for the first time by Nicolazzo et al. in 2016 (67), but remains unclear (Table 4). In most of the published series, the presence of PD-L1$^{(+)}$ CTCs at baseline was not associated with clinical outcomes, but 3 studies reported an increase in PD-L1$^{(+)}$ CTCs at tumor progression detection (58, 59, 63). Further studies are needed to clarify the predictive value of this CTC subpopulation.

In summary, CTC monitoring could be an early predictor of patients who will benefit from immunotherapy and serve as a complementary tool to radiologic assessment for therapeutic decision-making (Fig. 1). As the technical heterogeneity in CTC characterization clearly affects the clinical outcomes (32), the main challenge is to define standard operating procedures for CTC detection: technology and anti-PD-L1 antibody used. Moreover, the optimal threshold to predict the treatment response has not been determined yet. It seems that dichotomizing between CTC presence vs absence at baseline is more discriminating than using a threshold based on the median CTC number in the cohort. The ongoing LIBIL trial (NCT02511288), a French prospective study on 900 patients, aims to evaluate ctDNA and CTC predictive value on treatment

Table 5. Ongoing interventional studies to assess the predictive value of ctDNA level and bTMB for the response to ICIs in patients with advanced NSCLC.

Biomarker	Patients, n	Treatment	End date	Clinical trial name and/or number
ctDNA	60	Chemotherapy or immunotherapy	February 2021	WHENII, NCT03481101
ctDNA	100	Chemotherapy, targeted therapy, or immunotherapy	February 2023	ELUCID, NCT03926260
ctDNA + circulating immune cells	300	Immunotherapy	May 2024	LIBERTY LUNG, NCT04790682
bTMB	440	Atezolizumab versus chemotherapy	April 2024	BFAST, NCT03178552, COHORT C
bTMB	118	Pembrolizumab plus chemotherapy	November 2021	KEYNOTE-782, NCT03664024
bTMB + circulating immune cells	100	Nivolumab	May 2020	NCT04082988
bTMB	100	Atezolizumab	June 2022	BUDDY, NCT04059887

response in a dedicated cohort of patients with NSCLC treated by immunotherapy.

Limitations of Current Studies and Future Direction

Published studies indicate that ctDNA level and CTCs may have clinical utility for predicting the response to ICIs in patients with advanced NSCLC. Although the level of evidence is low (i.e., studies with small sample size and not always with a prospective design), overall, the studies highlight the prognostic impact of these 2 biomarkers in NSCLC. Their predictive value cannot be considered to be only ICI-specific. Indeed, in 94 NSCLC patients of the OAK trial, a high ctDNA level at 6 weeks was associated with poorer OS either in atezolizumab or docetaxel groups, and CTC presence at baseline has been recognized as a negative prognostic factor in 550 NSCLC patients mostly treated by chemotherapy (30, 68). However, ctDNA and CTC kinetics during immunotherapy can be easily used to predict the tumor response or treatment failure and survival, particularly when they are combined (22). For instance, radiologic assessment of the objective response can be hindered by the development of pseudoprogression, and CTC and/or ctDNA kinetics could help to distinguish this phenomenon from true progression (28). Alama et al. showed that in patients with radiological progression who continued immunotherapy because of a clear clinical benefit, the combined presence of high ctDNA levels and CTC number increased the risk of death by a factor of 8 compared with low or absent ctDNA levels/CTC number (22). However, the clinical impact of an early therapeutic switch on the basis of ctDNA/CTCs remains unknown and needs to be evaluated in a dedicated trial.

It has been suggested that ctDNA and CTCs could also help to decide the immunotherapy duration in patients with durable clinical benefit. Indeed, Hellmann et al. found that undetectable ctDNA during treatment was associated with longer event-free survival in 31 patients who had not progressed at 12 months from immunotherapy initiation (25). As the optimal ICI duration in responding patients remains unknown, a trial to assess treatment alleviation in patients with undetectable ctDNA could be proposed.

PD-L1$^{(+)}$ CTC presence at baseline is a negative prognostic factor in various cancers, including NSCLC (69). However, its predictive value for patients with NSCLC treated by ICIs has not been demonstrated, although PD-L1$^{(+)}$ CTCs tend to increase at progression time. The ongoing ALCINA trial (NCT04025541), in which PD-L1$^{(+)}$ CTCs are monitored during anti-PD-(L)1 therapy, could help to determine how this CTC subpopulation can predict the response or resistance to these agents. Several large observational studies are currently investigating the predictive value of PD-L1$^{(+)}$ CTCs in advanced NSCLC (Immunopredict NCT02827344, NCT04490564).

Blood TMB, which was initially considered very promising, remains a controversial biomarker of immunotherapy efficacy in NSCLC, notably because of the negative results of the B-F1RST and NEPTUNE

trials. The performance of bTMB may be improved by adjusting its value relative to the allele frequency, as suggested by Wang et al. (24), but this needs to be further investigated.

The ongoing interventional trials on ctDNA or bTMB are listed in Table 5. For example, the BFAST trial (NCT03178552) is a phase II/III multicohort study to evaluate the safety and efficacy of targeted therapies or immunotherapy in patients with advanced NSCLC harboring oncogenic somatic mutations or high TMB, identified by 2 blood-based NGS ctDNA assays. Analysis of the high bTMB cohort, in which patients are randomized between atezolizumab and chemotherapy, will help to clarify the role of this biomarker.

One of the limitations of the biomarkers presented in this review is linked to the high percentage of patients with NSCLC in whom one or more biomarkers cannot be detected (i.e., 9%–50% of patients without detectable CTCs at baseline and lack of specific ctDNA mutations in tumors without oncogenic driving mutations). To overcome this issue, the value of combining different liquid biopsy-based biomarkers (70), assessed before and during immunotherapy, should be investigated.

Specific mutations in different genes, such as *STK11* and *KEAP1*, are predictors of resistance to ICIs in NSCLC (35, 71). These mutations can be detected and quantified in ctDNA in a noninvasive manner. Aggarwal et al. evaluated specific genetic mutations in addition to bTMB using a 500-gene NGS panel in 66 patients with NSCLC who received first-line pembrolizumab-based treatment (40). The group found that a bTMB ≥16 mut/Mb had a positive predictive value (PFS and OS) only in the absence of *STK11*/*KEAP1*/*PTEN* or *HER2* exon 20 mutations.

Among the other potential predictive liquid biopsy-based biomarkers of ICI response, circulating immune cells and extracellular vesicles (e.g., exosomes) are currently under investigation. Han et al. collected blood samples from 40 patients with NSCLC who received anti PD-(L)1 antibodies, and observed longer PFS in patients with high TCR diversity in PD-1$^{(+)}$ CD8^{+} lymphocytes before treatment and in patients with increased TCR clonality after immunotherapy (72). PD-L1$^{(+)}$ exosomes also have been evaluated, with discordant results. Gunasekaran et al. suggested that a decrease of PD-L1$^{(+)}$ exosomes (after 8 weeks of treatment vs pretreatment) predicted better clinical outcomes (PFS and OS) in 25 patients treated with PD-(L)1 inhibitors (73). Conversely, another group showed that a fold change ≥1.86 in PD-L1$^{(+)}$ exosomes after 2 months of immunotherapy was associated with better efficacy in 20 patients with NSCLC (74). Future studies will precisely determine the value of PD-L1$^{(+)}$ exosomes. However, since the different groups used different methods to enrich and detect exosomes and to analyze PD-L1 expression, data cannot be compared. Therefore, it should be important to define guidelines for exosome identification and characterization and to standardize the operating procedures.

Summary

The use of liquid biopsy is rapidly growing in the field of cancer immunotherapy. Based on an overview of the potential interest of cf/ctDNA, bTMB, and CTCs for predicting ICI efficacy in patients with advanced NSCLC, ctDNA, or/and CTC monitoring during treatment could help to tailor therapeutic choices. The main limitations for their use are represented by the lack of standardization in detection methods and operating procedures. This standardization and the clinical implementation of liquid biopsy are the aim of the International Liquid Biopsy Standardization Alliance and the European Liquid Biopsy Society (75). More interventional studies are needed to demonstrate the clinical utility of these promising biomarkers (70).

Nonstandard Abbreviations: PD-L1, programmed death ligand 1; PD-1, programmed death 1; ctDNA, circulating tumor DNA; CTC, circulating tumor cell; NSCLC, nonsmall cell lung cancer; TKIs, tyrosine kinase inhibitors; ICIs, immune checkpoint inhibitors; EGFR, epidermal growth factor receptor; cfDNA, cell-free DNA; NGS, next-generation sequencing; MSAF, maximum somatic allele frequency; VAF, variant allele frequency; OS, overall survival; PFS, progression-free survival; TMB, tumor mutational burden; mut/Mb, mutations per megabase; tTMB, tissue tumor mutational burden; bTMB, blood tumor mutational burden; PD-L1$^{(+)}$ CTCs, PD-L1-positive CTCs.

Human Genes: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *ALK*, Anaplastic lymphoma kinase; *EGFR*, Epidermal growth factor receptor; *STK11*, Serine/threonine kinase 11; *KEAP1*, Kelch like ECH associated protein 1; *PTEN*, Phosphatase and tensin homolog; *HER2*, Erb-B2 receptor tyrosine kinase 2.

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References

1. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med 2015;373:1627–39.
2. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. N Engl J Med 2016;375:1823–33.
3. Gandhi L, Rodríguez-Abreu D, Gadgeel S, Esteban E, Felip E, Angelis FD, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. N Engl J Med 2018;2:258–85.
4. Herbst RS, Baas P, Kim DW, Felip E, Pérez-Gracia JL, Han JY, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet 2016;387:1540–50.
5. Rittmeyer A, Barlesi F, Waterkamp D, Park K, Ciardiello F, von Pawel J, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet 2017;389:255–65.
6. Berti A, Bortolotti R, Dipasquale M, Kinspergher S, Prokop L, Grandi G, et al. Meta-analysis of immune-related adverse events in phase 3 clinical trials assessing immune checkpoint inhibitors for lung cancer. Crit Rev Oncol Hematol 2021;162:103351.
7. Skoulidis F, Li BT, Dy GK, Price TJ, Falchook GS, Wolf J, et al. Sotorasib for lung cancers with KRAS p.G12C mutation. N Engl J Med 2021;384:2371–81.
8. Désage AL, Léonce C, Swalduz A, Ortiz-Cuaran S. Targeting KRAS mutant in non-small cell lung cancer: novel insights into therapeutic strategies. Front Oncol 2022;12:796832.
9. Zou Y, Hu X, Zheng S, Yang A, Li X, Tang H, et al. Discordance of immunotherapy response predictive biomarkers between primary lesions and paired metastases in tumours: a systematic review and meta-analysis. EBioMedicine 2021;63:103137.
10. Alix-Panabières C, Pantel K. Liquid biopsy: from discovery to clinical application. Cancer Discov 2021;11:858–73.
11. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. N Engl J Med 2018;379:1754–65.
12. Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res 2014;20:1698–705.
13. Wang X, Liu Y, Meng Z, Wu Y, Wang S, Jin G, et al. Plasma EGFR mutation abundance affects clinical response to first-line EGFR-TKIs in patients with advanced non-small cell lung cancer. Ann Transl Med 2021;9:635.
14. Behel V, Chougule A, Noronha V, Patil VM, Menon N, Singh A, et al. Clinical utility of liquid biopsy (cell-free DNA) based EGFR mutation detection post treatment initiation as a disease monitoring tool in patients with advanced EGFR-mutant NSCLC. Clin Lung Cancer 2022;23:410-18.
15. Cabel L, Riva F, Servois V, Livartowski A, Daniel C, Rampanou A, et al. Circulating tumor DNA changes for early monitoring of anti-PD1 immunotherapy: a proof-of-concept study. Ann Oncol 2017;28:1996–2001.
16. Iijima Y, Hirotsu Y, Amemiya K, Ooka Y, Mochizuki H, Oyama T, et al. Very early response of circulating tumour-derived DNA in plasma predicts efficacy of nivolumab treatment in patients with non-small cell lung cancer. Eur J Cancer 2017;86:349–57.
17. Goldberg SB, Narayan A, Kole AJ, Decker RH, Teysir J, Carriero NJ, et al. Early assessment of lung cancer immunotherapy response via circulating tumor DNA. Clin Cancer Res 2018;24:1872–80.
18. Giroux Leprieur E, Herbretau G, Dumenil C, Julie C, Giraud V, Labrune S, et al. Circulating tumor DNA evaluated by Next-Generation Sequencing is predictive of tumor response and prolonged clinical benefit with nivolumab in advanced non-small cell lung cancer. OncoImmunology 2018;7:e1424675.
19. Raja R, Kuziora M, Brohawn PZ, Higgs BW, Gupta A, Dennis PA, et al. Early reduction in ctDNA predicts survival in patients with lung and bladder cancer treated with durvalumab. Clin Cancer Res 2018;24:6212–22.
20. Chae YK, Davis AA, Agte S, Pan A, Simon NI, Iams WT, et al. Clinical implications of circulating tumor DNA tumor mutational burden (ctDNA TMB) in non-small cell lung cancer. Oncologist 2019;24:820–8.
21. Anagnostou V, Forde PM, White JR, Niknafs N, Hruban C, Naidoo J, et al. Dynamics of tumor and immune responses during immune checkpoint blockade in non-small cell lung cancer. Cancer Res 2019;79:1214–25.
22. Alama A, Coco S, Genova C, Rossi G, Fontana V, Tagliamento M, et al. Prognostic relevance of circulating tumor cells and circulating cell-free DNA association in metastatic non-small cell lung cancer treated with nivolumab. J Clin Med 2019;8:1011.
23. Li L, Wang Y, Shi W, Zhu M, Liu Z, Luo N, et al. Serial ultra-deep sequencing of circulating tumor DNA reveals the clonal evolution in non-small cell lung cancer patients treated with anti-PD1 immunotherapy. Cancer Med 2019;8:7669–78.
24. Wang Z, Duan J, Wang G, Zhao J, Xu J, Han J, et al. Allele frequency-adjusted blood-based tumor mutational burden as a predictor of overall survival for patients with NSCLC treated with PD-(L)1 inhibitors. J Thorac Oncol 2020;15:556–67.
25. Hellmann MD, Nabet BY, Rizvi H, Chaudhuri AA, Wells DK, Dunphy MPS, et al. Circulating tumor DNA analysis to assess risk of progression after long-term response to PD-(L)1 blockade in NSCLC. Clin Cancer Res 2020;26:2849–58.
26. Chen Y, Li X, Liu G, Chen S, Xu M, Song L, et al. ctDNA concentration, MIKI67 mutations and hyper-progressive disease related gene mutations are prognostic markers for camrelizumab and apatinib combined multiline treatment in advanced NSCLC. Front Oncol 2020;10:1706.
27. Ricciuti B, Jones G, Severgnini M, Alessi JV, Recondo G, Lawrence M, et al. Early plasma circulating tumor DNA (ctDNA) changes predict response to first-line pembrolizumab-based therapy in non-small cell lung cancer (NSCLC). J Immunother Cancer 2021;9:e001504.
28. Thompson JC, Carpenter EL, Silva BA, Rosenstein J, Chien AL, Quinn K, et al. Serial monitoring of circulating tumor DNA by next-generation gene sequencing as a biomarker of response and survival in patients with advanced NSCLC receiving pembrolizumab-based therapy. JCO Precis Oncol 2021;5:PO.20.00321.
29. Park CK, Oh HJ, Kim MS, Koh BG, Cho HJ, Kim YC, et al. Comprehensive analysis of blood-based biomarkers for predicting immunotherapy benefits in patients with

advanced non-small cell lung cancer. Transl Lung Cancer Res 2021;10:2103–17.
30. Zou W, Yaung SJ, Fuhlbrück F, Ballinger M, Peters E, Palma JF, et al. ctDNA predicts overall survival in patients with NSCLC treated with PD-L1 blockade or with chemotherapy. JCO Precis Oncol 2021;5: 827–38.
31. van der Leest P, Hiddinga B, Miedema A, Aguirre Azpurua ML, Rifaela N, ter Elst A, et al. Circulating tumor DNA as a biomarker for monitoring early treatment responses of patients with advanced lung adenocarcinoma receiving immune checkpoint inhibitors. Mol Oncol 2021;15:2910–22.
32. Mondelo-Macía P, García-González J, León-Mateos L, Anido U, Aguín S, Abdulkader I, et al. Clinical potential of circulating free DNA and circulating tumour cells in patients with metastatic non-small-cell lung cancer treated with pembrolizumab. Mol Oncol 2021;15: 2923–40.
33. Ren S, Chen J, Xu X, Jiang T, Cheng Y, Chen G, et al. Camrelizumab plus carboplatin and paclitaxel as first-line treatment for advanced squamous non-small-cell lung cancer (CameL-sq): a phase 3 trial. J Thorac Oncol 2022;17:544–57.
34. Ready N, Hellmann MD, Awad MM, Otterson GA, Gutierrez M, Gainor JF, et al. First-line nivolumab plus ipilimumab in advanced non-small-cell lung cancer (CheckMate 568): outcomes by programmed death ligand 1 and tumor mutational burden as biomarkers. J Clin Oncol 2019;37:992–1000.
35. Rizvi H, Sanchez-Vega F, La K, Chatila W, Jonsson P, Halpenny D, et al. Molecular determinants of response to anti-programmed cell death (PD)-1 and anti-programmed death-ligand 1 (PD-L1) blockade in patients with non-small-cell lung cancer profiled with targeted next-generation sequencing. J Clin Oncol 2018;36:633–41.
36. Hellmann MD, Paz-Ares L, Caro RB, Zurawski B, Kim SW, Costa EC, et al. Nivolumab plus ipilimumab in advanced non-small-cell lung cancer. N Engl J Med 2019;381:2020–31.
37. Paz-Ares L, Langer CJ, Novello S, Halmos B, Cheng Y, Gadgeel SM, et al. LBA80 – Pembrolizumab (pembro) plus platinum-based chemotherapy (chemo) for metastatic NSCLC: tissue TMB (tTMB) and outcomes in KEYNOTE-021, 189, and 407. Ann Oncol 2019;30:v917–8.
38. Fabrizio D, Malboeuf C, Lieber D, Zhong S, He J, White E, et al. Analytic validation of a next generation sequencing assay to identify tumor mutational burden from blood (bTMB) to support investigation of an anti-PD-L1 agent, atezolizumab, in a first line non-small cell lung cancer trial (BFAST). Ann Oncol 2017;28:v27.
39. Gandara DR, Paul SM, Kowanetz M, Schleifman E, Zou W, Li Y, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. Nat Med 2018;24: 1441–8.
40. Aggarwal C, Thompson JC, Chien AL, Quinn KJ, Hwang WT, Black TA, et al. Baseline plasma tumor mutation burden predicts response to pembrolizumab-based therapy in patients with metastatic non-small cell lung cancer. Clin Cancer Res 2020;26:2354–61.
41. Wang Z, Duan J, Cai S, Han M, Dong H, Zhao J, et al. Assessment of blood tumor mutational burden as a potential biomarker for immunotherapy in patients with non-small cell lung cancer with use of a next-generation sequencing cancer gene panel. JAMA Oncol 2019;5:696–702.
42. Herbst RS, Giaccone G, de Marinis F, Reinmuth N, Vergnenegre A, Barrios CH, et al. Atezolizumab for first-line treatment of PD-L1-selected patients with NSCLC. N Engl J Med 2020;383:1328–39.
43. Ma Y, Li Q, Du Y, Cai J, Chen W, Zhao G, et al. Blood tumor mutational burden as a predictive biomarker in patients with advanced non-small cell lung cancer (NSCLC). Front Oncol 2021;11:640761.
44. Rizvi NA, Cho BC, Reinmuth N, Lee KH, Luft A, Ahn MJ, et al. Durvalumab with or without tremelimumab vs standard chemotherapy in first-line treatment of metastatic non-small cell lung cancer: the MYSTIC phase 3 randomized clinical trial. JAMA Oncol 2020;6:661–74.
45. Chen X, Fang L, Zhu Y, Bao Z, Wang Q, Liu R, et al. Blood tumor mutation burden can predict the clinical response to immune checkpoint inhibitors in advanced non-small cell lung cancer patients. Cancer Immunol Immunother 2021;70:3513–24.
46. Socinski M, Velcheti V, Mekhail T, Chae YK, Leal TA, Dowell JE, et al. Final efficacy results from B-F1RST, a prospective phase II trial evaluating blood-based tumour mutational burden (bTMB) as a predictive biomarker for atezolizumab (atezo) in 1L non-small cell lung cancer (NSCLC). Ann Oncol 2019;30:v919–20.
47. AstraZeneca. Update on the Phase III NEPTUNE trial of Imfinzi plus tremelimumab in Stage IV nonsmall cell lung cancer. https://www.astrazeneca.com/mediacentre/press-releases/2019/update-onthe-phase-iii-neptune-trial-of-imfinzi-plustremelimumab-in-stage-iv-nonsmall-celllung-cancer-21082019.html (Accessed October 2022).
48. Wang J, Lu S, Hu C, Sun Y, Yang K, Chen M, et al. 1264P Updated analysis of tislelizumab plus chemotherapy vs chemotherapy alone as first-line treatment of advanced squamous non-small cell lung cancer (SQ NSCLC). Ann Oncol 2020;31: S817.
49. Garassino MC, Gadgeel SM, Rodriguez-Abreu D, Felip E, Esteban E, Speranza G, et al. Evaluation of blood TMB (bTMB) in KEYNOTE-189: pembrolizumab (pembro) plus chemotherapy (chemo) with pemetrexed and platinum versus placebo plus chemo as first-line therapy for metastatic nonsquamous NSCLC. J Clin Oncol 2020;38:9521.
50. Jiang T, Chen J, Xu X, Cheng Y, Chen G, Pan Y, et al. On-treatment blood TMB as predictors for camrelizumab plus chemotherapy in advanced lung squamous cell carcinoma: biomarker analysis of a phase III trial. Mol Cancer 2022;21:4.
51. Paz-Ares L, Ciuleanu TE, Cobo M, Schenker M, Zurawski B, Menezes J, et al. 98O First-line nivolumab (NIVO)+ipilimumab (IPI)+2 cycles chemotherapy (chemo) vs 4 cycles chemo in advanced non-small cell lung cancer (aNSCLC): association of blood and tissue tumor mutational burden (TMB) with efficacy in CheckMate 9LA. J Thorac Oncol 2021;16:S750–1.
52. Nabet BY, Esfahani MS, Moding EJ, Hamilton EG, Chabon JJ, Rizvi H, et al. Noninvasive early identification of therapeutic benefit from immune checkpoint inhibition. Cell 2020;183:363–376.e13.
53. Eslami-S Z, Cortés-Hernández LE, Cayrefourcq L, Alix-Panabières C. The different facets of liquid biopsy: a kaleidoscopic view. Cold Spring Harb Perspect Med 2020;10:a037333.
54. Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, et al. Frequent expression of PD-L1 on circulating breast cancer cells. Mol Oncol 2015;9:1773–82.
55. Jacot W, Mazel M, Mollevi C, Pouderoux S, D'Hondt V, Cayrefourcq L, et al. Clinical correlations of programmed cell death ligand 1 status in liquid and standard biopsies in breast cancer. Clin Chem 2020;66: 1093–101.
56. Sinoquet L, Jacot W, Gauthier L, Pouderoux S, Viala M, Cayrefourcq L, et al. Programmed cell death ligand 1-expressing circulating tumor cells: a new prognostic biomarker in non-small cell lung cancer. Clin Chem 2021;67:1503–12.
57. Dhar M, Wong J, Che J, Matsumoto M, Grogan T, Elashoff D, et al. Evaluation of PD-L1 expression on vortex-isolated circulating tumor cells in metastatic lung cancer. Sci Rep 2018;8:1–10.
58. Guibert N, Delaunay M, Lusque A, Boubekeur N, Rouquette I, Clermont E, et al. PD-L1 expression in circulating tumor cells of advanced non-small cell lung cancer patients treated with nivolumab. Lung Cancer 2018;120:108–12.
59. Janning M, Kobus F, Babayan A, Wikman H, Velthaus JL, Bergmann S, et al. Determination of PD-L1 expression in circulating tumor cells of NSCLC patients and correlation with response to PD-1/PD-L1 inhibitors. Cancers (Basel) 2019;11:835.
60. Tamminga M, de Wit S, Hiltermann TJN, Timens W, Schuuring E, Terstappen LWMM, et al. Circulating tumor cells in advanced non-small cell lung cancer patients are associated with worse tumor response to checkpoint inhibitors. J Immunother Cancer 2019;7:173.
61. Castello A, Carbone FG, Rossi S, Monterisi S, Federico D, Toschi L, et al. Circulating tumor cells and metabolic parameters in

NSCLC patients treated with checkpoint inhibitors. Cancers (Basel) 2020;12:487.
62. Papadaki MA, Sotiriou AI, Vasilopoulou C, Filika M, Aggouraki D, Tsoulfas PG, et al. Optimization of the enrichment of circulating tumor cells for downstream phenotypic analysis in patients with non-small cell lung cancer treated with anti-PD-1 immunotherapy. Cancers (Basel) 2020;12:1556.
63. Ikeda M, Koh Y, Teraoka S, Sato K, Oyanagi J, Hayata A, et al. Longitudinal evaluation of PD-L1 expression on circulating tumor cells in non-small cell lung cancer patients treated with nivolumab. Cancers (Basel) 2021;13:2290.
64. Dall'Olio FG, Gelsomino F, Conci N, Marcolin L, De Giglio A, Grilli G, et al. PD-L1 expression in circulating tumor cells as a promising prognostic biomarker in advanced non-small-cell lung cancer treated with immune checkpoint inhibitors. Clin Lung Cancer 2021;22:423–31.
65. Zhang L, Zhang X, Liu Y, Zhang T, Wang Z, Gu M, et al. PD-L1+ aneuploid circulating tumor endothelial cells (CTECs) exhibit resistance to the checkpoint blockade immunotherapy in advanced NSCLC patients. Cancer Lett 2020;469:355–66.
66. Hosokawa M, Kenmotsu H, Koh Y, Yoshino T, Yoshikawa T, Naito T, et al. Size-based isolation of circulating tumor cells in lung cancer patients using a microcavity array system. PLoS One 2013;8:e67466.
67. Nicolazzo C, Raimondi C, Mancini M, Caponnetto S, Gradilone A, Gandini O, et al. Monitoring PD-L1 positive circulating tumor cells in non-small cell lung cancer patients treated with the PD-1 inhibitor nivolumab. Sci Rep 2016;6:31726.
68. Lindsay CR, Blackhall FH, Carmel A, Fernandez-Gutierrez F, Gazzaniga P, Groen HJM, et al. EPAC-lung: pooled analysis of circulating tumour cells in advanced non-small cell lung cancer. Eur J Cancer 2019;117:60–8.
69. Ouyang Y, Liu W, Zhang N, Yang X, Li J, Long S. Prognostic significance of programmed cell death-ligand 1 expression on circulating tumor cells in various cancers: a systematic review and meta-analysis. Cancer Med 2021;10:7021–39.
70. Alix-Panabières C. The future of liquid biopsy. Nature 2020;579:S9.
71. Marinelli D, Mazzotta M, Scalera S, Terrenato I, Sperati F, D'Ambrosio L, et al. KEAP1-driven co-mutations in lung adenocarcinoma unresponsive to immunotherapy despite high tumor mutational burden. Ann Oncol 2020;31:1746–54.
72. Han J, Duan J, Bai H, Wang Y, Wan R, Wang X, et al. TCR Repertoire diversity of peripheral PD-1+CD8+ T cells predicts clinical outcomes after immunotherapy in patients with non-small cell lung cancer. Cancer Immunol Res 2020;8:146–54.
73. Gunasekaran M, Russo A, Cardona AF, Perez DdM, Lapidus R, Cooper B, et al. Exosomal PD-L1 expression as non-invasive biomarker for immune checkpoint inhibitors in non-small cell lung cancer. J Immunol 2020;204:90.10.
74. Yang Q, Chen M, Gu J, Niu K, Zhao X, Zheng L, et al. Novel biomarkers of dynamic blood PD-L1 expression for immune checkpoint inhibitors in advanced non-small-cell lung cancer patients. Front Immunol 2021;12:665133.
75. Connors D, Allen J, Alvarez JD, Boyle J, Cristofanilli M, Hiller C, et al. International Liquid Biopsy Standardization Alliance White Paper. Crit Rev Oncol Hematol 2020;156:103112.