



Current and future biomarkers for outcomes with immunotherapy in non-small cell lung cancer

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Abstract: Immune checkpoint inhibitors (ICI) have been validated as an effective new treatment strategy in several tumoral types including lung cancer. This remarkable shift in the therapeutic paradigm is in large part due to the duration of responses and long-term survival seen with ICI. However, despite this, the majority of cancer patients do not experience benefit from ICI. Even among patients who initially respond to ICI, disease progression may ultimately occur. Moreover, in some patients, these drugs may be associated with new patterns of progression such as pseudo-progression and hyper-progressive disease, and different toxicity profiles with immune-related adverse events. Therefore, the identification of predictive biomarkers may help to select those patients most likely to obtain a true benefit from these drugs, and avoid exposure to potential toxicity in patients who will not obtain clinical benefit, while also reducing the economic impact. In this review, we summarize current and promising potential predictive biomarkers of ICI in patients with non-small cell lung cancer (NSCLC), as well as pitfalls encountered with their use and areas of focus to optimize their routine clinical implementation.

Keywords: Biomarker; immune checkpoint inhibitor (ICI); immunotherapy; non-small cell lung cancer (NSCLC); liquid biopsy

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Introduction

Programmed death-ligand 1 (PD-L1) expressed in tumor cells remains the only predictive biomarker used in daily clinical practice for patients with advanced non-small cell lung cancer (NSCLC). However, the tumoral heterogeneity of PD-L1 expression represents a bias that raises challenges

for widespread use of this biomarker. Tumor mutational burden (TMB), defined as the number of somatic mutations contributing to the tumor's immunogenicity, has also been reported as a potential predictive biomarker. However, one of the major limitations for the use of biomarkers in NSCLC is the paucity of tumor tissue from biopsies (1), which severely limits the number and type of tests that

can be performed, and alternative biomarkers and ways of evaluating biomarkers are actively being sought. Liquid biopsies, mainly dedicated to the analysis of circulating tumor DNA (ctDNA), are a novel non-invasive tool for genomic profiling and assessment of tumor heterogeneity in NSCLC (1). In this review we provide an overview of available biomarkers in NSCLC in tissue and liquid biopsies. We focus on tumor-related biomarkers, notably PD-L1 and TMB, and also investigate biomarkers related to the tumor microenvironment (TME), notably tumor infiltrating lymphocytes (TILs), and host-derived biomarkers including circulating immune cells, soluble markers and the gut microbiome.

Tumor-related biomarkers

PD-L1

The increasing comprehension of the PD-1/PD-L1 axis has been a major step in the development of tumoral immunotherapy. As more PD-(L)1 inhibitors are developed, the research focus has moved to identifying biomarkers for the activation of this axis, both in tumoral cells as well as immune cells and subsequently on any circulating counterparts (Figures 1,2).

Tissue PD-L1

The development of immune checkpoint inhibitor (ICI) targeting PD-(L)1 in NSCLC has been closely followed by the assessment of potential predictive biomarkers. PD-L1 expression on tumor cells as determined by immunohistochemistry represents the most valuable and unique predictive biomarker in daily clinical practice. Response rates (RR) and outcome in terms of overall survival (OS) increase with higher PD-L1 expression (2-4). However various pitfalls remain, several of which have been thoroughly explored, notably PD-L1 tumoral expression, with technical issues related to the development of different monoclonal antibodies used for testing (5), differences in the choice of cut-off threshold, as well as a range of biological issues; these include the fact that PD-L1 is differentially expressed with inconsistent predictive values across tumor types (6) and furthermore that expression may be modulated during treatment (7). Moreover, the predictive value may depend on the mechanism of PD-L1 induction. Oncogenic-addicted NSCLC may cause an intrinsic elevation of PD-L1 expression and can be associated with a worse prognosis (8). On the other hand, extrinsic induction of PD-L1 by INF- γ

produced by lymphocytes seems to be associated with a favorable predictive value (9).

In the first-line treatment setting of advanced NSCLC patients, PD-L1 expression by immunohistochemistry on tumor cells has been widely used to select and stratify patients considered most likely to obtain benefit from ICI. However, not all pivotal trials have achieved a survival benefit with ICI compared with standard platinum-based chemotherapy (10-14). In contrast to nivolumab, which did not report an OS benefit (10,11), atezolizumab (14) and pembrolizumab (12,13) did, especially in tumors with high PD-L1 expression (12-14). These latter observations lead to FDA approval of pembrolizumab in tumors with PD-L1 $\geq 1\%$ and atezolizumab in tumors with PD-L1 expression $\geq 50\%$ in tumor cells, whereas the EMA only approved pembrolizumab as monotherapy in the first-line setting in tumors with PD-L1 expression $\geq 50\%$. However, with the availability of more recent first-line data reporting the efficacy of ICI plus chemotherapy combination therapy compared with chemotherapy alone, the predictive value of PD-L1 expression appears diminished. These studies demonstrated that all patients derived a survival benefit with the combination compared to chemotherapy alone, regardless of PD-L1 expression and histologic subtype (15-17). This led to FDA/EMA approval for ICI plus chemotherapy regardless of the level of PD-L1 expression. In the CheckMate 227 trial, nivolumab plus ipilimumab reported improvement in OS compared with chemotherapy in PD-L1 negative tumors [17.2 versus 12.2 months, HRT 0.64, 95% confidence interval (CI): 0.51–0.81], however, efficacy of the combination in this subset of patients was only an exploratory analysis (18).

In PACIFIC trial, although durvalumab improved PFS and OS in PD-L1 $\geq 1\%$ tumors, in 148 patients with PD-L1 $< 1\%$ tumors durvalumab neither improved PFS (HR 0.73; 95% CI: 0.48–1.11) nor OS (HR: 1.14, 95% CI: 0.71–1.84). Of note, efficacy of durvalumab in PD-L1 negative tumors was an exploratory *post-boc* analysis requested by EMA and the enrollment in the trial was not restricted to any threshold level for PD-L1 expression, and PD-L1 status was not mandatory for inclusion (19).

Soluble PD-L1

Surface molecules can assume two forms of expression, membrane-bound protein and a soluble form generated after proteolytic cleavage, such as is the case for the immune checkpoint molecules PD-1, PD-L1 and CTLA-4. Elevated serum concentrations of the soluble forms

	Biomarker		Method of detection	Tissue type	Association with positive predictive value	Level of evidence* and results	
Tumor-related	PD-L1 pathway	Tissue PD-L1	Immunohistochemistry	Tumor	Positive PD-L1 tumor expression	Concordant results	
		Soluble PD-L1	ELISA chemiluminescence	Blood	Conflicting results	Conflicting results	
	Antigen recognition	MSI-MDRD	Immunohistochemistry	Tumor	Mismatch repair deficiency: hypermutator phenotypes	Concordant results	
		TMB	Tumoral TMB	WES; NGS (Foundation One CDx™ and MSK-IMPACT)	Tumor	High mutational rate (tTMB≥175 mut/exome)	Conflicting results
			Blood TMB	WES; NGS	Blood	High mutational rate	Conflicting results
		Tumor-specific genotype	Direct sequencing; NGS	Tumor	Absence of EGFR, ALK or ROS1 mutations	Conflicting results	
	Others	CTC	Enrichment (CellSearch®) and detection (IF staining)	Blood	Low baseline CTC count	Results to be validated	
		ctDNA	Multiplex targeted NGS; digital droplet PCR; RNASeq; SNP array	Blood	Undetectable ctDNA levels at week 8 of treatment; early decrease in ctDNA burden at 1 month of treatment	Concordant results	
		Epigenetic	Bisulfite conversion of genomic DNA, whole-genome amplification and array-based capture and scoring of CpG loci	Tumor	EPIMMUNE signature	Results to be validated	
Tumor/host interaction: microenvironment	TILs		Immunohistochemistry; flow cytometry	Tumor	CD3 ⁺ CD8 ⁺ infiltration; TILs density>5%	Concordant results	
	B cells and TILs		Immunohistochemistry; flow cytometry	Tumor	TLS presence	Results to be validated	
Host-related	Gene expression signature		Microarray analysis	Tumor	IFN-γ gene signature	Concordant results	
	Circulating immune cells	CD3 ⁺ T cells	Complete blood count; flow cytometry	Blood	Expansion of PD-1 ⁺ CD8 T cells during treatment; low baseline proportion of CD28 ⁺ CD57 ⁺ KLRG1 ⁺ CD8 T cells	Results to be validated	
		Neutrophils and MDSC	Complete blood count; flow cytometry	Blood	Low baseline proportion of circulating M-MDSCs; low baseline dNLR	Results to be validated	
	Soluble systemic immune or inflammatory markers	LDH	Spectrophotometry	Blood	Low baseline LDH levels	Conflicting results	
		CRP	Immunoturbidimetry	Blood	Low baseline CRP levels	Results to be validated	
		Albumin	Immunoturbidimetry	Blood	High baseline albumin levels	Results to be validated	
		Cytokines	ELISA chemiluminescence	Blood	Early decrease of IL-8 during treatment; increase of TNF-α and IFN-γ during treatment	Results to be validated	
	Microbiota		Bacterial 16S ribosomal RNA gene sequencing	Gut microbiota	High gut diversity at baseline Akkermansia enrichment	Results to be validated	

Prospective study 1	Concordant results A
Retrospective study with independent cohorts 2	Conflicting results B
Retrospective study without a control group 3	Results to be validated in further studies C

Figure 1 Main biomarkers of clinical outcomes in NSCLC patients treated by anti-PD-(L)1 antibody and technical requirements. *, level of evidence color code; yellow: prospective study; Red: retrospective study with independent cohorts; Blue: retrospective study without control. ALK, anaplastic lymphoma kinase; CRP, C-reactive protein; CTC, circulating tumor cells; (ct)DNA, (circulating tumor) deoxyribonucleic acid; dNLR, derived neutrophil-to lymphocyte ratio; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunoassay; IF, immunofluorescence; IFN-γ, interferon gamma; IL, interleukin; LDH, lactate dehydrogenase; MDSC, myeloid-derived suppressive cells; NGS, next-generation sequencing; RNA, ribonucleic acid; TILs, tumor-infiltrating lymphocytes; TLS, tertiary lymphoid structures; (t)TMB, (tumoral) tumor mutational burden; TNF-α, tumor necrosis factor alpha; WES, whole exome sequencing.

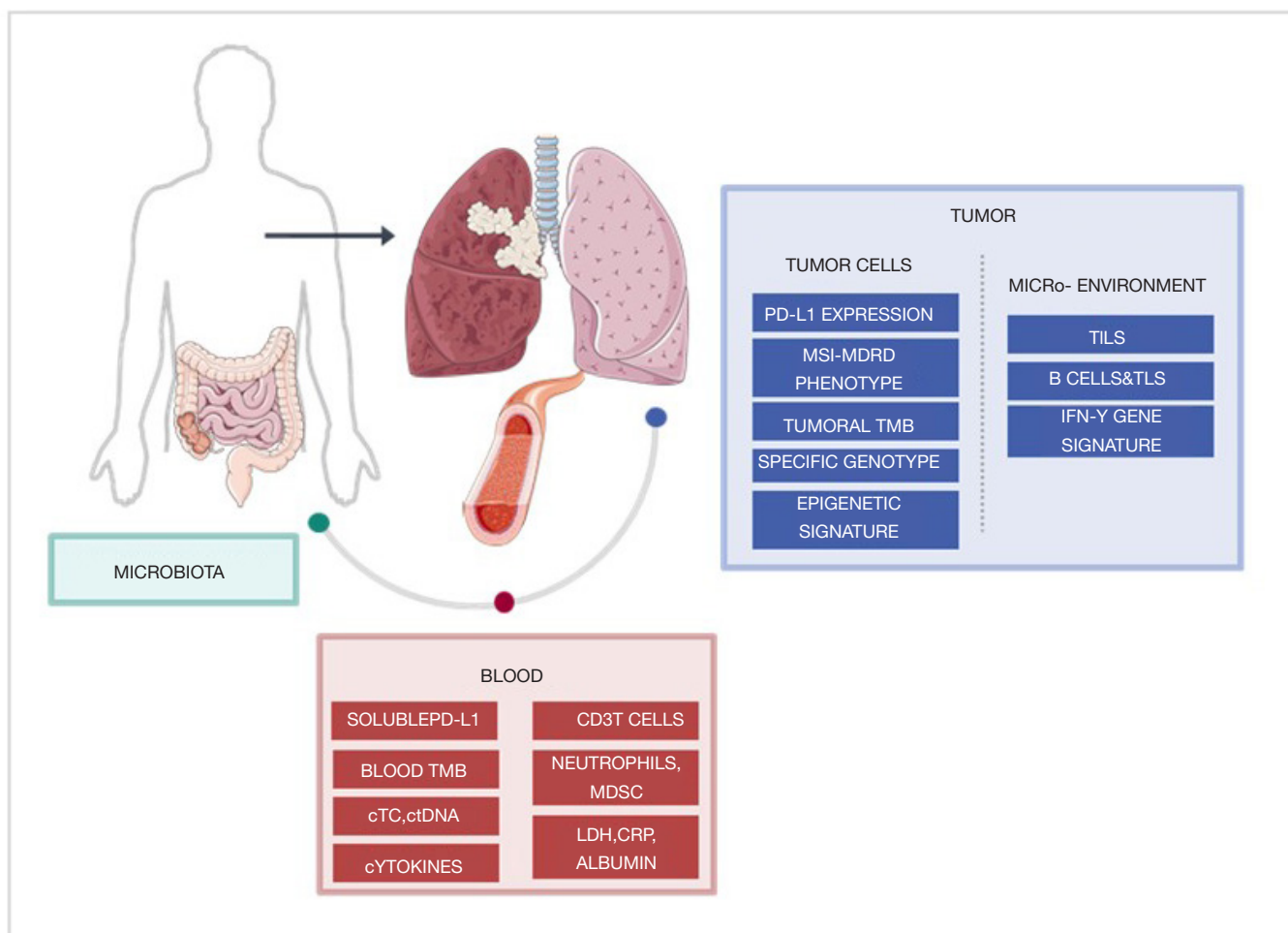


Figure 2 Main biomarkers of clinical outcomes in NSCLC patients treated by anti-PD-(L)1 antibody. CRP, C-reactive protein; CTC, circulating tumor cells; (ct)DNA, (circulating tumor) deoxyribonucleic acid; IFN- γ , interferon gamma; LDH, lactate dehydrogenase; MDSC, myeloid-derived suppressive cells; TILS, tumor-infiltrating lymphocytes; TLS, tertiary lymphoid structures; (t)TMB, (tumoral) tumor mutational burden.

of these three molecules (sPD-1, sPD-L, and sCTLA-4, respectively) were initially associated with autoimmune diseases (20) and are now being explored as predictive biomarkers in cancer (21).

In cultured cell lines, sPD-1 is produced by effector T cells and myeloid cells (22) and production of the soluble form is related to PD-L1 expression levels (23). Nevertheless, a correlation was not described between tumor PD-L1 expression (immunohistochemistry analysis on tumoral cells) and sPD-L1 levels (ELISA analysis) in patients with non-thoracic malignancies such as diffuse large B-cell lymphomas and renal cell carcinomas (24). These results hints at the preponderant role of the TME,

including immune non-malignant cells, in sPD-L1 production (24,25).

In advanced NSCLC patients, higher sPD-L1 or sPD-L2 expression was reported compared to healthy controls, and high sPD-L1 expression significantly correlated with worse prognosis (26-28), as has been reported in other malignancies (29,30). In a recent study, the role of baseline and dynamic evolution of sPD-1 and sPD-L1 after two cycles of nivolumab were assayed in 51 advanced NSCLC patients (31). Positivity was defined as a plasma level above the lower limit of quantification (0.156 ng/mL). A composite criteria (sCombo) was defined by sPD-L1 and/or sPD-1 positivity. Score positivity at baseline was associated

with shorter median progression-free survival [PFS; 78 days, 95% CI: 55–109 versus 658 days, 95% CI: 222–not reached; hazard ratio (HR) 4.12, 95% CI: 1.95–8.71; $P=0.0002$] and OS (HR 3.99, 95% CI: 1.63–9.80; $P=0.003$). In multivariate analyses, score positivity remained significantly associated with shorter PFS (HR 2.66, 95% CI: 1.17–6.08; $P=0.02$). Results from another NSCLC cohort treated with nivolumab support the negative prognostic impact of sPD-L1 plasma level at baseline under ICI (32). However, contradictory results have been published regarding the predictive value of dynamic sPD-L1 expression (25,31). Several splicing variants have been described of sPD-L1, some of them lacking transmembrane domain. These variants may act as “decoys” of PD-L1 antibody *in vitro* and in animal model and may participate to PD-L1 antibody resistance (33).

Other potential sources of PD-L1 include exosomes which are small membrane vesicles (diameter, 50–100 nm) with an endocytic origin, released by many cell types including T cells and dendritic cells. Tumor-derived exosomes contain substantial amounts of biologically active proteins, including immune checkpoint proteins (34). In patients with lung cancer, the amount of PD-L1 in these vesicles can impair immune functions. Adding patient PD-L1⁺ exosomes to autologous lymphocytes can reduce *in vitro* cytokine production and induce T lymphocyte CD8⁺ apoptosis (34). In a mouse xenograft model, exosomal PD-L1 promoted a tumor immune escape mechanism that was abolished by PD1/PD-L1 blockers. Baseline exosomal PD-L1 may also be used as a prognostic factor (35).

These results highlight that the PD-1/PD-L1 axis remains a cornerstone of PD(L)1 inhibition biomarkers. To date, PD-L1 expressed by tumoral cells on a biopsy remains the only biomarker used in clinical practice in the context of administering ICI therapies. Nevertheless, it does not fully explain the sensitivity and resistance mechanisms to ICI, in particular for combination therapy. Circulating PD-L1 offers additional information, but prospective validation is warranted.

MMRD and TMB

Another major aspect of ICI response prediction is the ability of the immune system to detect specific tumoral antigens. ICI have met with success in tumor types with high mutational load including NSCLC, melanoma and tumors associated with DNA mismatch repair (MMR), suggesting the potential of mutational burden as a response biomarker. This led to the assessment of tumoral

antigenicity, mainly via TMB evaluation in both tissue and blood samples (*Figures 1,2*).

MSI and MMRD tumors

DNA MMR is a system of recognition and repair of mutations arising during DNA replication. Mismatch repair deficiency (MMRD) has been associated with hypermutator phenotypes such as microsatellite instability (MSI) (36). This instability can lead to an increased TMB and high mutational load (36). Consequently, MSI and MMRD tumors are highly immunogenic and this has been used as a predictive biomarker for ICI outcome in clinical trials. These trials have consistently reported a highly favorable RR in several MSI-high (MSI-H) and MMRD tumor types (37,38), leading to the first therapeutic agnostic approvals by the FDA in patients with unresectable or metastatic MSI-H or MMRD solid tumors, independent of the tumor localization. Nevertheless, this condition is rare in lung cancer (<1%) (39). Some deleterious alterations of *POLE*, *POLD1* and *MSH2* genes have been found in patients with high TMB responding to ICI (40), however mutation signatures in lung cancer have been shown to be mostly associated with smoking habits (41).

Recently, DNA damage response and repair (DDR) gene alterations were reported in tumoral tissues of 50% of a cohort of NSCLC patients (42). Median TMB was significantly higher in the DDR-positive group compared to the DDR-negative group (12.1 versus 7.6 mut/Mb, $P<0.001$), and DDR-positive tumors had a significantly higher RR (30.3% versus 17.2%, $P=0.01$), longer median PFS (5.4 versus 2.2 months, HR: 0.58, 95% CI: 0.45–0.76, $P<0.001$), and longer median OS (18.8 versus 9.9 months, HR: 0.57, 95% CI: 0.42–0.77, $P<0.001$) with PD-(L)1 therapy, after adjusting for PD-L1, TMB, performance status, tobacco use, and line of therapy.

Tumoral tissue and blood-based TMB

Recent data suggest that TMB could be another predictor of ICI efficacy. TMB is calculated as the total number of non-synonymous somatic mutations of the genomics coding area. These mutations can lead to neoantigen formation and contribute to the immunogenicity of the tumor. The type of mutation may also contribute to the resulting antigenicity (43), with insertions and deletions leading to more antigen formation than non-synonymous single nucleotide variants. The TMB can be evaluated with various techniques (Whole exome sequencing, Whole Genome Sequencing, Next

Generation Sequencing), with different thresholds, and can be performed using tumor tissue (tTMB) or blood (bTMB). Data on their predictive value are conflicting, particularly in terms of ICI combinations and ICI chemotherapy combinations.

Tumoral TMB

The first-line trial CheckMate 026 compared nivolumab monotherapy versus chemotherapy in PD-L1 $\geq 5\%$ NSCLC. In exploratory subgroup analyses according to TMB, higher RR and longer PFS were reported with nivolumab compared with chemotherapy in tumors with high TMB (RR: 47% versus 28%; PFS: 9.7 versus 5.8 months) (10). Of note, no difference in OS was found (18.3 versus 18.8 months). Following these results, the TMB was used prospectively as a biomarker for PFS as one of the two coprimary endpoints in the CheckMate 227 study. This study compared the combination of nivolumab and ipilimumab to platinum-based chemotherapy (44) in the first-line setting in advanced NSCLC. In the group of patients with ≥ 10 mut/Mb (high tTMB; N=299, 17% of the randomized population), the combination arm reported a significant benefit compared with chemotherapy in PFS (7.2 versus 5.5 months, HR 0.58, 75% CI: 0.41–0.81) as well as an improvement in RR (45.3% versus 26.9%) (44). The coprimary endpoint of the study (OS in PD-L1 $\geq 1\%$) was also met, with improved OS for the combination compared to chemotherapy (17.2 versus 12.2 months, HR 0.62; 95% CI: 0.48–0.78), leading to FDA approval of the nivolumab plus ipilimumab combination in the first-line setting in advanced NSCLC patients with PD-L1 $\geq 1\%$. However, this HR benefit was subsequently shown to occur regardless of PD-L1 expression (PD-L1 $\geq 1\%$; HR 0.79, 95% CI: 0.65–0.96; and in PD-L1 $< 1\%$; HR 0.62, 95% CI: 0.49–0.79), or TMB level (high: HR 0.68, 95% CI: 0.51–0.91; or low: HR 0.75, 95% CI: 0.59–0.94). Consequently, these results do not support tTMB as a predictive biomarker for ICI combination (11).

Similarly the efficacy of pembrolizumab plus platinum-based chemotherapy versus chemotherapy alone in the first-line setting for metastatic NSCLC, occurred regardless of tTMB status assessed by whole exome sequencing, and defining high tTMB as ≥ 175 mut/exome (45). Therefore, randomized controlled trials have so far failed to show a survival benefit when stratifying patients by tTMB and these data do not currently support the prognostic or predictive value of tTMB in NSCLC patients.

Blood-based TMB

First-line tTMB can be challenging to obtain, and use of blood-based TMB (bTMB) is increasing, with several studies suggesting its predictive role. The OAK and POPLAR studies comparing ICI versus chemotherapy in the second-line setting (46), used several bTMB cutoff points ≥ 10 , ≥ 16 , and ≥ 20 mut/Mb, and blood-based approaches (measured by the Foundation Medicine assay) to assess TMB. Both studies reported an inverse relationship between TMB and OS HR, suggesting that bTMB may predict benefit of atezolizumab as second-line therapy in NSCLC (47).

In the first-line setting, the MYSTIC trial assessed the efficacy of a durvalumab plus tremelimumab combination or durvalumab monotherapy compared with platinum-based chemotherapy in a PD-L1 expressing population (PDL-1 $\geq 25\%$). This study did not meet any of the three primary endpoints: PFS and OS for the immunotherapy combination compared to chemotherapy and OS for durvalumab compared to chemotherapy. However in a retrospective exploratory analysis, patients with high bTMB [≥ 16 mut/Mb (48) or ≥ 20 mut/Mb] derived a survival benefit with combined durvalumab plus tremelimumab compared with chemotherapy (49). Similarly, the non-randomized phase II B-FIRST study evaluated bTMB as a predictive biomarker for atezolizumab according to a cut-off of 16 mut/Mb. Atezolizumab achieved longer PFS (5.0 versus 3.5 months, HR 0.80, 90% CI: 0.54–1.18) and OS (23.9 versus 13.4 months, HR 0.66, 90% CI: 0.40–1.10) in tumors with bTMB ≥ 16 mut/Mb versus < 16 mut/Mb, respectively. These results suggest a clinical benefit, albeit not statistically significant, of atezolizumab in tumors with high bTMB (50). The ongoing phase III BFAST trial evaluating different ICIs according to bTMB in metastatic NSCLC (NCT03178552) is designed to address this question.

Similarly, in another retrospective study with 66 advanced NSCLC, first-line treatment with pembrolizumab either alone or in combination with chemotherapy achieved longer PFS in patients with bTMB ≥ 16 mut/Mb than in those with bTMB < 16 mut/Mb (14.1 versus 4.7 months; HR 0.30, 95% CI: 0.16–0.60; $P < 0.001$). Median OS for patients with bTMB ≥ 16 mut/Mb was not reached versus 8.8 months for patients with bTMB < 16 mut/Mb (HR 0.48, 95% CI: 0.22–1.03; $P = 0.061$). However, the predictive role of bTMB did not apply for patients with co-mutations in *STK11/KEAP1/PTEN* and *ERBB2* (51).

On the other hand, the phase III NEPTUNE trial (NCT02542293) comparing durvalumab and tremelimumab versus platinum-based chemotherapy in the first-line treatment did not meet the OS endpoint in patients with a high bTMB (≥ 20 mut/Mb) (52).

To date, the prognostic and predictive value of tTMB or bTMB remains a challenge, mainly due to the absence of uniform standards for the cut-off point for defining high TMB, sample type and the technical platform used to evaluate it. Data from prospective trials are warranted to more accurately identify the predictive and/or prognostic value of TMB for benefit.

Specific tumor mutations and circulating DNA

Along with TMB, the tumoral genome has further potential to be used as a predictive marker. Specific tumor genotypes such as oncogene addicted or *LKB1/STK11* mutated tumors have been hypothesized to be associated with primary resistance ICI. Moreover, direct evaluation of tumoral cell DNA and circulating DNA have also been investigated as possible circulating biomarkers (Figures 1,2).

Tumor-specific genotype

Some genomic alterations have been correlated with lack of efficacy of ICI. In a multicentric international retrospective cohort of 551 oncogenic addicted NSCLC patients (IMMUNOTARGET), objective RRs according to driver alterations following anti-PD(L)-1 treatment were *KRAS* 26%, *BRAF* 24% *ROS1* 17%, *MET* 16%, *EGFR* 12%, *HER2* 7%, *RET* 6%, and *ALK* 0% (8). Median PFS in the overall cohort was 2.8 months. However, in another retrospective study (N=107), the RRs were 26% for *BRAF*-V600, 33% for *BRAF*-nonV600, 27% *HER2*, 38% *MET* and 38% *RET*-altered, similar to outcomes reported in wild-type NSCLC patients (53).

Somatic mutations and co-mutations have also been associated with specific type of TME and ICI resistance, such as is the case for inactivating *LKB1/STK11* genomic alterations. These alterations are present in ~25% of *KRAS*-mutant adenocarcinomas and are frequently associated with a “cold”, non-T cell-inflamed microenvironment with a paucity of infiltrating CD3⁺, CD4⁺ and CD8⁺ T cells and low tumor cell expression of PD-L1. *KEAP1* is also associated with a cold micro-environment in particular when it is associated with a *PTEN* mutation (54). Furthermore, co-mutations in either *LKB1/STK11* or

KEAP1 were associated with worse clinical outcomes with chemoimmunotherapy using pemetrexed-carboplatin (or cisplatin) plus the anti-PD-1 antibody pembrolizumab in non-squamous NSCLC (55). Similarly, in the MYSTIC trial, the occurrence of *LKB1/STK11* or *KEAP1* mutations correlated with poorer PFS and OS across treatment arms compared with wild-type counterparts (56). In contrast, an exploratory analysis of the phase III KEYNOTE 042 trial (pembrolizumab versus chemotherapy in the first-line setting of PD-L1 $\geq 1\%$ advanced NSCLC patients) the occurrence of *KEAP1* or *LKB1/STK11* mutation (34% of the whole population) did not affect the efficacy of pembrolizumab (RR 31%, OS 18 months) (57). However, the sample size of this study is too small to impact daily clinical practice with ICI as monotherapy as the standard treatment in this population with these co-mutations, and further formal analyses are warranted.

Circulating tumor cells (CTC) and circulating-tumor DNA

To overcome the difficulties associated with obtaining tissue, non-invasive methods have been developed in the search for ICI biomarkers, including analysis of CTC and ctDNA in NSCLC. CTC and ctDNA have been used for assessing PD-L1 status (58,59) and as dynamic biomarkers of ICI efficacy (59-61). In NSCLC patients, obtaining ctDNA clearance at one (62) or two months (63) after ICI initiation has been found to correlate with a prolonged duration of response. In contrast, an increase in ctDNA $>20\%$ at 6 weeks after nivolumab was associated with a worse outcome (63). PD-L1 can be assessed on CTCs, however sensitivity ranges from 45% (59) to 93% (58) of samples. To date, no prospective trial has confirmed PD-L1 expression on CTCs as a predictive biomarker of ICI efficacy (58,59,64-66). On the contrary, some studies suggest that high PD-L1 expression on CTCs at baseline was associated with a poor outcome in patients treated with anti-PD(L)1 (58). Moreover, PD-L1⁺ CTC increases at progression, possibly predicting resistance to ICI (65). Finally, logistically, cost and turnaround time of these analyses are critical and may limit the clinical applicability of CTC analyses.

Epigenetic biomarkers

DNA methylation is an epigenetic chemical “flag” which is critical for several cellular activities and is often altered in human diseases including cancer. A DNA methylation

profile signature was generated in a discovery cohort of 34 NSCLC patients treated with ICI (EPIMMUNE) (67). This signature was associated with an improved outcome (PFS, $P=0.0067$; OS, $P=0.0012$), and was confirmed in an EPIMMUNE validation cohort with 47 patients. This methylation involves forkhead box P1 (*FOXP1*), and the unmethylated status of this single gene was confirmed as a predictive positive biomarker for PFS (HR 0.415, $P=0.006$) and OS (HR 0.409, 95% CI: 0.22–0.78; $P=0.009$) in a third cohort. The EPIMMUNE-positive signature was not associated with PD-L1 expression, the presence of CD8⁺ cells, or mutational load. Moreover, this study associated the signature status with tumoral inflammatory infiltration.

Tumor-host interaction: Micro-environment biomarkers

The TME is composed of immune cells, fibroblasts, and vascular and lymphatic tissues surrounding the tumoral cells. This microenvironment is constantly evolving, dependent on signaling molecules, with the cancer cells promoting immune evasion on the one hand and the host controlling tumoral proliferation on the other (68,69). Within the TME, several kinds of host immune cells can be recruited from adaptative cells (such as B and T lymphocytes) and innate immunity (such as polymorphonuclear leukocytes and Natural killer). The functional orientation of the immune population has been described as being dependent on the local immune contexture (70). It has been associated with patient outcomes in multiple tumor types and has been explored for its predictive potential when using ICI. Despite being more difficult to assess in clinical practice, mainly due to a lack of access to sufficient tissue samples, understanding the microenvironment is likely to be critical to deciphering the mechanism of immunotherapy (71) (Figures 1,2).

TILs

TILs have been correlated with improved survival in several cancer types including NSCLC (72). High levels of TILs, in particular CD8-positive TILs, correlate with improved survival probably reflecting a greater immune tumoral recognition by the immune system (73). This inflamed, ‘hot tumor’ phenotype, may have predictive value during ICI treatment (74). Exploratory studies have confirmed the potential interest of CD8⁺ infiltrate as a biomarker in several conditions. The immune infiltrate and PD-L1 tumoral

expression was associated with response to nivolumab in 65 advanced lung cancer patients (75). In another study enrolling 98 patients with advanced NSCLC, TIL density >5% correlated with PFS (HR 0.31, CI: 0.14–0.68, $P=0.004$) and higher objective RR (OR =3.5, 95% CI, 1.06–11.7, $P=0.04$) in a multivariate analysis (76). The nature of the T cell infiltrate may also predict therapeutic outcome with ICI. Anagnostou *et al.* described the oligoclonal expansion of pre-existing intratumoral T-cell clones in patients with tumoral response to ICI (61). Functionality and specific TIL phenotypes have been associated with response, such as high CD3 expression, T cell low granzyme B and low Ki-67 levels, proposed to be a “dormant” phenotype and associated with a better outcome (77).

B cells and tertiary lymphoid structures (TLS)

Recent studies suggest a role for B cells when localized to tumor compartments called TLS. TLS are aggregates of immune cells in response to immunological stimuli in the presence of B cells (78). TLS, like TILs, are considered to be a predictor of increased survival (79). This role may be related to B cell activation, antibody cell death, and cooperation with T cells. Apparition of TLS during treatment in various tumor types appears to be associated with favorable evolution during ICI treatment (80–82), although this is yet to be evaluated in lung cancer.

Several other microenvironment parameters including hypoxia, angiogenesis and the extracellular matrix are under exploration. To be successful, these parameters must be correlated with biological or radiological surrogates and may be candidates to be combined with existing biomarkers (71).

Gene expression signature

Multiple-gene signatures may have prognostic value in NSCLC (83). Nevertheless, limited data exist about the predictive role of immune gene signatures in NSCLC tumors treated with ICI. These signatures are mostly related to IFN γ signaling. In several malignancies, the baseline IFN γ mRNA signature had predictive role for RR and PFS with pembrolizumab (84). In NSCLC, PD-1 gene expression along with a 12-gene signature tracking CD8, CD4 T-cell, natural killer cells, and IFN activation was associated with nonprogressive disease and PFS (85). In the POPLAR trial comparing docetaxel to atezolizumab

in second-line advanced NSCLC patients, tumors with high expression of the T-effector-associated and IFN- γ -associated gene signature demonstrated improved OS (HR, 0.43; 95% CI, 0.24–0.77) (86). In another study in 17 patients with NSCLC treated with nivolumab, mRNA expression levels (divided in tertile, high if superior to the 1st tertile) were explored in tumoral samples. The only mRNA associated with PFS was IFN γ (5.1 versus 2.0 months, HR 6.66; 95% CI: 1.2–36.8, P=0.0297, for high and low expression respectively) (87).

Host related biomarkers: functional status of the immune system

Circulating immune cells

Circulating blood cells populations, including immune cells subpopulations may reflect host immune system functionality (Figures 1,2). Baseline and on-treatment variations in these populations have frequently been associated with cancer outcomes. Different techniques are available, some robust and clinically validated such as complete blood counts (CBC) and others more specific with subpopulation analysis using flow cytometry immunophenotyping. One strength of using fresh immune cell whole blood real-time monitoring, is that it allows more reliable data to be obtained for some brittle immune cell populations. Comparative studies with frozen peripheral blood mononuclear cells (PBMC) and fresh whole blood identified that some populations could be detected with the same sensitivity, while others may not be consistently determined from frozen PBMC. For example, for some memory T cell populations (using CD45RA and/or CD62L and/or CCR7 staining), regulatory T cells (Treg) seemed to be underestimated in PBMCs, with a large variability of 20% to 30%. In addition, neutrophils, the main subpopulation of leucocytes, cannot be recovered from frozen PBMCs (88).

Blood CD3⁺ T lymphocytes

Lymphocyte functional status, e.g., activation, senescence or polarization can be studied on circulating T lymphocytes and some populations have been investigated as biomarkers with ICI treatment. In NSCLC patients, PD-1⁺ T cells are more frequent than in healthy controls (27,89) and are associated with a worse clinical outcome. In a study by Zheng *et al.* including 42 NSCLC patients, median OS and

PFS were shorter in patients with high expression of PD-1⁺CD4⁺ circulating T cells (89). In another study, other checkpoint inhibitor molecules including PD-1, PD-L1 and PD-L2 on PBMC correlated with a worse prognosis. Among 70 patients who did not receive ICI, PD-L1 expression on CD8⁺ and PD-1 expression on CD4⁺ T cells were associated with poor outcome (27).

Evolution and proliferation of these populations can also be used for predicting ICI efficacy. Kamphorst *et al.* reported the dynamic evolution of immune checkpoints on T cells in a cohort of 29 NSCLC patients treated with ICI. An increase in proliferation of circulating PD-1⁺ CD8⁺ T cells within four weeks after treatment initiation was associated with better outcome (90). The phenotype of these cells has been elucidated with an effector phenotype (HLA-DR⁺, CD38⁺, Bcl-2^{low}), associated with expression of co-stimulatory molecules (CD28, CD27, ICOS) as well as high expression of CTLA-4. Early proliferation of PD-1⁺ CD8 T cells after PD-1 infusion was observed in more than three-quarters (78.5%) of patients who experienced clinical benefit versus only 21.5% of patients with progressive disease (90).

Another study associated the proliferative response of peripheral PD-1⁺CD8⁺ T cells after 1 week of anti-PD-1 therapy with a positive outcome in patients with NSCLC (91). Ki-67_{D7/D0} was tested as a predictive biomarker in patients with NSCLC (N=79) or thymic epithelial tumors (N=31). These T cells were found to have proliferated seven days after ICI with a reduction after three weeks (91). The cut-off was optimized to 2.8 for Ki-67_{D7/D0} with a higher probability of clinical benefit in patients with Ki-67_{D7/D0} \geq 2.8 than in patients with Ki-67_{D7/D0} <2.8 (P<0.001). The same trend was found with PFS, which was 8.7 months (95% CI: 4.3–13.2 months) in patients with Ki-67_{D7/D0} \geq 2.8 and 3.9 months (95% CI: 1.2–6.6 months) in those with Ki-67_{D7/D0} <2.8 (P=0.027), without modification of its predictive value when adding a score using tumoral PD-L1 expression (91).

T cell differentiation phenotypes have also been explored as a biomarker for ICI treatment. Patients with metastatic NSCLC express more memory effectors and fewer naïve T cells than control patients (92). In a cohort of 22 NSCLC patients, central memory (CM) to effector memory (EM) T cell ratio (TCM/TEM) was correlated with response during ICI treatment (92), as well as with longer PFS. It was also associated with high PD-L1 expression in the tumor and an increased inflammatory signature. In another cohort (N=51), the level of baseline functional CD4⁺ memory T cells, in

particular those with low co-expression of PD-1/LAG-3, were associated with response under ICI (93).

Immunosenescence is another phenotype related to chronic antigenic stimulation. T cell senescence reflects a terminal differentiation status of a T cell with low proliferative activity, an oligoclonal T cell receptor repertoire, and reduced capacity to recognize antigenic diversity (94). Senescent and exhausted T-cells share some characteristics, however senescent cells conserved their cytotoxic potential (95). Blood senescent T cells, defined as CD3⁺CD8⁺CD28^{meq}CD57⁺KLRG1⁺, have recently been studied in patients with advanced NSCLC treated with ICI (96). Senescent populations were more frequent in NSCLC patients and in patients receiving chemotherapy compared to treatment-naïve patients (97). In a recent preliminary study, 43 ICI treated patients were evaluated for T cell senescence. Patients presenting a high proportion of senescent CD8⁺ T cells had significantly lower RR (31% versus 0%, $P=0.03$), disease control rate (81% versus 29%, $P=0.002$), PFS [7.3 months, 95% CI: 2.7–non reached (NR) versus 1.8 months, 95% CI: 1.3–NR; $P=0.02$] and OS (NR, 95% CI: 6.04–NR, versus 2.6 months, 95% CI: 1.9–NR; $P=0.01$). Interestingly senescent CD8⁺ T cells were not associated with clinical outcome in a cohort of patients treated with chemotherapy.

CD3⁺ cells are major but heterogenous effectors of antitumoral immunity. Deciphering T cell subpopulations such as senescent, central memory or activated PD1⁺ may provide a better understanding of immunotherapy mechanisms and predictive tools, however this requires confirmation in independent cohorts.

Circulating neutrophils and myeloid-derived suppressive cells (MDSC)

An association between neutrophils and poor prognosis has been suspected in several cancer types for some time. Recent literature indicates that tumors may play a role in early differentiation of neutrophils by creating various phenotypic and functional polarization states able to influence tumor development. In solid tumors, neutrophils can be found both in the TME and the blood, and are generally associated with a poor prognosis (98). Neutrophils dominate the NSCLC immune landscape with a mostly immune suppressive role (99). Recently we demonstrated that some circulating innate immune markers including neutrophils were related to prognosis in advanced NSCLC patients (100). Neutrophils increase may be mostly related

to granulocyte colony-stimulating factor production by the tumor (101). Several ratios have been evaluated as potential biomarkers, including neutrophil to lymphocyte ratio (NLR; neutrophils/lymphocytes) and derived neutrophil to lymphocyte ratio [dNLR: (neutrophils)/(leucocytes-neutrophils)] (98). In a large meta-analysis including over 40,000 patients, NLR >4 was associated with worse OS, cancer-specific survival, PFS and disease-free survival in all types and stage of cancers (102). However, the threshold of these ratios has not been homogenously determined across published reports and these studies were mainly conducted in the pre-ICI area.

We previously evaluated the combined dNLR and lactate dehydrogenase (LDH) level as the lung immune prognostic index (LIPI) in advanced NSCLC patients treated with immunotherapy. Patients with high baseline dNLR (>3) and LDH (above the upper limit of normal) were associated with worse prognosis for ICI treatment ($N=466$), but not with chemotherapy ($N=152$) (103). Nonetheless, the prognostic value of NLR and LIPI in NSCLC patients treated with ICI remains uncertain. In two recent pooled cohorts from clinical trials enrolling 1,489 (104) and 2,440 patients (105), treated with ICI or chemotherapy, a good LIPI score was associated with better OS both in patients receiving ICIs and in those receiving chemotherapy. Questions remain over differences in the magnitude of the benefit for patients treated with immunotherapy (106), and the role of the LIPI score warrants evaluation in prospective trial. Dynamic evolution of the LIPI score during treatment with ICI has also been correlated as a prognostic factor (107).

Other immunoregulatory cells can be recruited during chronic inflammatory processes such as MDSC. This cell population is more frequent in patients with lung cancer than in healthy volunteers (108) and has been associated with worse prognosis (109). It is a heterogeneous population and can be mainly divided into two groups, neutrophil-like (g-MDSC or PMN-MDSC) and monocyte-like (M-MDSC) (108,110-112). Increased levels of M-MDSCs have been correlated with worse PFS following chemotherapy (3 versus 9 months, $P<0.01$) (113) and RR ($P=0.02$) (114). Concerning ICI treatment, a recent study in two prospective cohorts including 63 patients evaluated the role of Lox-1⁺ PMN-MDSC and Treg cells (115). No difference between responders and progressors was found at baseline, however after a single nivolumab administration, Lox-1⁺ PMN-MDSC diminished in responding patients. An

inverse correlation was observed for Tregs. The elevation of the Treg to Lox-1⁺PMN-MDSCs ratio (TRM ratio, cutoff 0.39) was associated with longer median PFS (103 versus 35 days; $P=0.0079$) than in patients with low TRM. We consider that PMN-MDSC proliferation and recruitment in non-responders after anti-PD-1 therapy might impair its efficacy (115).

Soluble systemic markers: LDH, CRP, albumin and other inflammatory proteins

Systemic nonspecific inflammation or metabolic shifts may be involved in immune-resistance mechanisms during cancer development. Some generic blood tests, many of which are validated in daily practice, have been investigated as potential inflammatory biomarkers in cancer patients.

Glucose metabolism is impaired in cancer cells with predominant glycolysis despite aerobic conditions (known as the Warburg effect). This allows rapid proliferation but requires upregulation of most enzymes involved in the glycolytic pathway, including LDH. LDH elevations had already been associated with an adverse prognosis in several studies before the emergence of ICI, in several types of solid tumors (116), including thoracic malignancies (117). The question of the predictive role of LDH during ICI treatment has previously been evoked; in a meta-analysis with advanced NSCLC patients treated with ICI, high pre-treatment LDH levels (above the upper limit of normal) were significantly correlated with shorter PFS (HR 1.62, 95% CI: 1.26–2.08, $P<0.001$) and OS (HR 2.38, 95% CI: 1.37–4.12, $P=0.002$) (118).

Systemic inflammation and nutritional status biomarkers have been investigated regarding ICI efficacy, likely with less specificity but wide availability in routine practice. Retrospective studies have shown that C-reactive protein (CRP) elevation has been associated with worst prognosis in NSCLC and other malignancies (119,120). Consistently, in the non-randomized prospective B-FIRST trial evaluating atezolizumab monotherapy in advanced NSCLC, a decrease in serum CRP over six weeks was associated with PFS and OS benefits (50). Poor nutritional status with decreased albumin has been correlated with poor response to ICI, again in a retrospective study (121). Concerning cytokines, several interleukins have been associated with the disease course. In NSCLC, the dynamic evolution of IL-8 during ICI treatment was reported as a biomarker, with a benefit for patients with early decreases of IL-8 levels (122). In others studies, an increase of tumor necrosis

factor and INF- γ during ICI therapy correlated with better outcome (25,123).

Gut microbiome

The gut microbiome has been the subject of considerable interest during the last decade. It is suspected of playing a critical role in the maturation and education of the immune system at the basal state as well as during carcinogenesis with an inflammation induced by bacteria. Most studies have been conducted in metastatic melanoma patients with reports of enrichment of a given bacterial subpopulation [e.g., *Bifidobacterium* (124), or *Ruminococcaceae* (125)] or higher diversity (125) associated with response. In NSCLC patients under ICI, responders to nivolumab had higher gut diversity at baseline with a stable composition during treatment (126). Evolution under ICI has been correlated with specific microbiota compositions; a study in 60 NSCLC patients showed an enrichment of *Akkermansia muciniphila* at cancer diagnosis on responders (69%) compared to 34% on non-responders, and correlation with TH1 cytokine polarization (127). Microbiota diversity can also be affected by antibiotics and may have an impact on ICI therapy (128). While these results are interesting, confirmation in a prospective trial is currently ongoing.

Conclusions

To date, most PD(L)1 inhibition strategies in NSCLC have been based on PD-L1 expression on tumor cells. Other promising biomarkers such as TMB have not been adopted in routine clinical use, mainly due to the difficulty of differentiating between their prognostic versus their predictive value as well as technical or logistical global coherence in terms of methodology and thresholds. Several host-related or microenvironment-based biomarkers have recently been uncovered and deserve validation in independent cohorts in particular tumoral epigenetic signature and circulating immune cells subpopulation. All these biomarkers provide insight into the complexity of the antitumoral immune response. In the next few years, the challenge will likely be how to combine these predictive factors to accurately predict the response to ICI and personalize the choice of combination therapy.

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