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Review

Predictive biomarkers of response for immune checkpoint inhibitors in non–small-cell lung cancer

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Abstract Immune checkpoint blockade has been a pivotal development in the management of advanced non–small-cell lung cancer (NSCLC). Although durable antitumour activity and improved survival have been observed in a subset of patients, there is a need for additional predictive biomarkers to improve patient selection and avoid toxicity in potential non-responders. This review will address the use and limitations of tumour programmed death-ligand 1 expression as a predictive biomarker and review emerging biomarker strategies specifically related to NSCLC including genetic alterations (tumour mutation burden, loss and gain activated mutations), tumour-related factors (tumour microenvironment) and factors related to the host immune system. Novel approaches in biomarker detection such as peripheral blood monitoring will also be reviewed.

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1. Introduction

The advent of immune checkpoint inhibitors (CPIs) as both first- and second-line treatment for advanced non–small-cell lung cancer (NSCLC) results in improved survival and antitumour response compared with chemotherapy in selected patients. Unfortunately, up to 60% of patients with advanced NSCLC will not benefit from antiprogrammed death-1 (PD-1) or programmed death-ligand 1 (PD-L1) agents [1–4]. The need to discover and validate predictive biomarkers, beyond tumour PD-L1 expression, to better select patients who will derive most benefit and spare unnecessary toxicity and cost in non-responders remains an ongoing challenge.

At present, tumour PD-L1 expression is the only approved predictive biomarker for PD-(L)1 blockade in NSCLC. Even though PD-L1 expression is currently used to inform treatment decisions and regulatory approval, its expression may vary over time and by site among multiple tumour lesions [5]. Archival biopsy specimens collected months or years before starting treatment may not reflect the current expression status [6], particularly in pretreated patients whereby exposure to chemotherapy, radiotherapy and antiangiogenic therapy can upregulate PD-L1 expression [7,8]. Tumour PD-L1 expression is regulated by two main mechanisms: constitutive (intrinsic) expression and induced (extrinsic) by interferon gamma (IFN-γ) secreted by infiltrating lymphocytes [9]. Hence, in some situations, intrinsic elevated PD-L1 expression should correlate with worse differentiation and poorer prognosis; in contrast, expression induced by INF-y seems to be associated with a better prognosis [10]. Other activation mutations may also alter PD-L1 expression. For example, Janus kinase 3 (JAK3)-activating mutations increase the expression of PD-L1 in NSCLC [11,12].

Immunohistochemistry (IHC) is used to evaluate tumour PD-L1 expression. Table 1 summarises the five diagnostic PD-L1 assays developed for each anti–PD-1/PD-L1 agent (anti–PD-1: nivolumab and pembrolizumab and anti–PD-L1: atezolizumab, durvalumab and avelumab). These assays differ in their threshold of 'PD-L1 positivity' and approval as a companion or complementary assay. Blueprint 2, a phase IIA prospective study, evaluated the analytical comparability of these five assays concluding that three (28-8, 22C3 and SP263) of the five assays were comparable. The SP142 clone (which is used to score both tumour and immune cells) detects consistently less, whereas 73-10 is more sensitive, in PD-L1 positive detection [13,14]. Possibly because of dynamic expression and differences in diagnostic assays, the use of tumour PD-L1 expression is ultimately limited by its suboptimal negative predictive value. Response rates of 11–20% have been reported in patients with negative PD-L1 expression

Table 1
Different PD-L1 cut-offs and IHC clones across different CPIs and studies (data from Blueprint Project 1 [13] and 2 [14]).

Agent	Anti–PD-1 drugs		Anti–PD-L1 drugs		
	Nivolumab	Pembrolizumab	Atezolizumab	Durvalumab	Avelumab
IHC clone	22-8 (Dako)	22C3 (Dako)	SP142 (Ventana)	SP263 (Ventana)	73-10 (Dako)
PD-L1 cut-offs (%)	TC 1%, 5%, 10%	TPS 1%, 50%	TC 1%, 5%, 50%	IC 1%, 5%, 10%	TC 1%, 50%, 80%
FDA approval	Complementary test	Companion test	Complementary test	Waiting for approval	Waiting for approval
Comparability on PD-L1 positivity	Same detection with 22C3 and SP263	Same detection with 22–8 and SP263	Detects less	Same detection with 22–8 and 22C3	Detect more
Cut point above positive threshold (% of cases)	60.5% (PD-L1 ≥1%)	60.5% (PD-L1 ≥1%)	78.9% (TC1/IC1)	52.6% (PD-L1 ≥25%)	NA
Agreement between Pathologist	Similar for PD-L1 on tumour cells 80% agreement for immune cell staining				NA

PD-L1, programmed death ligand-1; PD-1, antiprogrammed death-1; IHC, immunohistochemistry; CPI, checkpoint inhibitors; FDA, Food and Drug Administration; TC, tumour cell; IC, immune cell; TPS, tumour proportional score; NA, not available.

[3,15,16]. Therapeutic strategies under investigation to increase response rates in PD-L1–negative patients include combination cytotoxic T-lymphocyte–associated antigen 4 (CTLA4)/PD-1 or PD-L1 blockade [17,18] and combination of CPI with chemotherapy in unselected patients [1–4,19].

2. Predictive biomarkers beyond PD-L1 expression

2.1. Tumour-related biomarkers

2.1.1. Tumour mutation burden

Clinical outcomes correlate with tumour mutation burden (TMB) in multiple cancers treated with CPI, including NSCLC [20–23]. TMB is the total number of non-synonymous somatic mutations of the genomic coding area. Germline mutations are excluded from the TMB as the host immune system recognises these as normal alterations [24]. Non-synonymous somatic mutations alter the amino acid sequence of proteins encoded by affected gene, forming neoantigens [20,22]. It is hypothesised that neoantigen formation contributes to the intrinsic immunogenicity of a tumour; however, it is unclear that which specific neoantigens drive this host immune response [25,26]. Preclinical data in melanoma and NSCLC indicate that high-load frameshift indel (insertion and deletion mutations) neoantigens are highly immunogenic compared with non-synonymous single nucleotide variant load and represent future areas of research, which means that not only the quantity but also the quality of mutations is decisive in generating immunogenic neoantigens [27].

High TMB is observed in greater frequency in cancers associated with mutagen exposure such as lung cancers and melanoma (TMB 7.2 mutation per megabase [mut/Mb] and 13.5 mut/Mb, respectively). In lung cancer, the level of TMB appears similar across all histologies. A low level of TMB has been reported in tumours harbouring driver mutations, (e.g. TMB $\geq$ 20 mut/Mb in about 1% of epidermal growth factor receptor [EGFR], anaplastic lymphoma kinase (ALK), ROS1 and MET mutant cancers) with the exception of BRAF and KRAS, where high TMB is reported in more than 10% [28].

However, as for PD-L1 expression, the thresholds that define high TMB level vary, and reported values also depend on the different techniques used [21,29].

Whole exome sequencing (WES) has traditionally been used to evaluate TMB; however, cost and result turnaround time limits its widespread use. Alternative approaches such comprehensive genome profiling based on next-generation sequencing (NGS) have been explored to measure TMB levels [30–32]. Use of TMB to predict response to CPI in advanced NSCLC is a promising biomarker as summarised in Table 2. For the first time, Rizvi et al.[21] reported that high non-synonymous mutation burden was associated with higher overall response rate (ORR, 59% versus 12%, $p = 0.01$), significantly longer progression-free survival (PFS; NR versus 3.4 m; hazard ratio [HR] 0.19, $p = 0.0004$) and grater durable clinical benefit (DCB), i.e. response lasting>6 months, (79% versus 18%, $p = 0.0011$) compared with low TMB in advanced NSCLC treated with pembrolizumab. Low TMB was identified as a negative predictor of response with only 12% (2/17) of patients with low TMB responding to pembrolizumab. The presence of molecular smoking signatures also correlated significantly with TMB. Kowanetz et al.[29] reported that high TMB was associated with improved ORR, PFS and overall survival (OS), independent of the PD-L1 status in three phase II second-line trials evaluating atezolizumab. Goodman et al.[32] also observed positive correlation between TMB and outcomes in NSCLC. The pattern of mutation burden (clonal versus subclonal) may further aid patient selection for CPI [33]. A further analysis of the same cohort from the study by Rizvi et al. showed a positive correlation between high clonal neoantigen burden and favourable response to pembrolizumab in lung adenocarcinoma; this was not observed in the squamous subtype, probably due to a lower expression of immune-regulatory genes such as human leucocyte antigen (HLA) class I genes and β2 microglobulin (β2M) in the squamous subtype. In this cohort, the only patient with high TMB who experienced progressive disease (relapsing after 2 months) was found to have >80% subclonal mutations [34].

Peters et al.[35] presented data from Checkmate 026 in patients with untreated advanced NSCLC with PD-L1 expression >1% treated with nivolumab. In 312 evaluable patients with high TMB ($\geq$13 mut/Mb), nivolumab improved median PFS (9.7 versus 5.8 months; HR, 0.62; 95% confidence interval [CI], 0.38 to 1.00) and ORR (46.8% versus 28.3%) compared with platinum-doublet chemotherapy. Furthermore, patients with a low and median TMB level fared better with chemotherapy (6.9 versus 4.1 months). At 18 months, almost 75% of patients with high TMB and high PD-L1 expression in the nivolumab arm were progression free compared with 25% of those with high TMB but lower PD-L1 expression. Less than 10% of low/medium TMB patients were progression free even if they had PD-L1 $\geq$ 50%. Rizvi et al.[36] recently reported an exploratory analysis from 848 patients with NSCLC. Of these, 240 patients were treated with either PD-1 or PD-L1 inhibitors, demonstrating a greater DCB ($p = 0.006$) and improvement in PFS and ORR in the high TMB subgroup. The analysis was conducted using the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets platform, which has been recently approved by the Food and Drug Administration (FDA) to measure the TMB level. Furthermore, in a cohort of 49 patients, a good correlation was found

Table 2
Correlation of TMB with clinical outcomes in NSCLC.

Author/study phase	No	Agents	High TMB cut-offs	Technique	ORR/DCB (%) TMB high versus low	p-value	mPFS (months) TMB high versus low	mOS (months) TMB high versus low
Rizvi et al., 2015 Retrospective [21]	34 NSCLC	Pembrolizumab	≥178 mut/Mb	WES	ORR:59 versus 12% DCB:79 versus 18%	p = 0.01 p = 0.0011	NR versus 3.4 HR 0.19 p = 0.0004	NA
Kowantez et al., 2017 BIRCH + FIR Phase II [29]	102 first-line 371 second-line NSCLC (PD-L1 selected)	Atezolizumab	≥13.5 mut/Mb first-line ≥17.1 mut/Mb second-line	FM-CGP FM-CGP	ORR: 28% versus 13% ORR: 29% versus 16%	NA	HR 0.54 first line HR 0.5 second line	HR 0.45 first line HR 0.7 second line
Kowantez 2017 POPLAR Phase II [29]	92 second-line NSCLC (PD-L1 unselected)	Atezolizumab CHT	≥15.8 mut/Mb second-line	FM-CGP	ORR: 20% versus 8%	NA	HR 0.49	HR 0.5
Campesato et al., 2016 Retrospective [30].	34 NSCLC[1]	Pembrolizumab	≥7 mut/Mb ≥13 mut/Mb	FM-CGP HSL-CGP	DCB both panel 69% versus 20%	p = 0.01	14.5 versus 3.4 HR 0.27[2] p = 0.005 HR 0.29[3] P = 0.008	NA
Goodman et al., 2017 Retrospective [32]	36 NSCLC + 52 melanoma	MonoIO ComboIO	≥20 mut/Mb −	NGS	44% versus 5%	p = 0.0023	5.7 versus 1.9 p = 0.0023	NR versus 8 p = 0.0791
McGranahan et al., 2016 Retrospective [34]	34 NSCLC[1]	Pembrolizumab	CNB-high	WES	NA	NA	HR 0.20* p = 0.0017	p = 0.061**
Peters et al., 2017 Checkmate 026 Phase III [35]	312 first-line NSCLC	Nivolumab CHT	≥13 mut/Mb	WES	TMB-high, ORR: 46.8% (nivo) versus 28.3% (CHT)	NA	9.7 versus 4.1 (nivo) 5.8 versus 6.9 (CHT)	OS ~ same
Rizvi et al., 2018 Retrospective [36]	49 NSCLC	Anti−PD-1/anti−PD-L1	−	NGS and WES	DCB: 38.6 versus 25.1%	p < 0.001	longer PFS HR: 1.38 p = 0.024	NA
Hellman et al., 2018 Checkmate 227 Phase III [37]	139 versus 160 first-line NSCLC	Nivolumab + ipilimumab CHT	>10 mut/Mb	FM	TMB-high, ORR: 45.3% (nivo + ipi) versus 26.9% (CHT)		TMB-high: 7.5 (nivo + ipi) versus 5.5 (CHT) (HR 0.58, p < 0.001)	NA
Roszik et al., 2016 Retrospective [38]	29 NSCLC	Pembrolizumab	High PTML ≥100	WES	Low PTML ORR: 0%	NA	8.3 versus 4.1 p = 0.0003	NA

TMB, tumour mutational burden; NSCLC, non−small-cell lung cancer; ORR, overall response rate; DCB, durable clinical benefit (partial or stable response lasting >6 months); mPFS, median progression-free survival; mOS, median overall survival; HR, hazard ratio; mut/Mb, mutations per megabase; WES, whole exome sequencing; FM-CGP, Foundation Medicine comprehensive gene profiling; HSL-CGP, Institutional Panel comprehensive gene profiling; NGS, next-generation sequencing; PTML, predictive tumour mutational load; CNB, clonal neoantigen burden; ITH, intra-tumour heterogeneity; CHT, chemotherapy; MonoIO, monoimmunotherapy; ComboIO, combination of two immunotherapies; PD-L1, programmed death ligand 1; NR, not reached; NA, not available.
[1]Using Rizvi et al. cohort.
[2]Using FM-CGP panel.
[3]Using HSL-CGP.
*Using ITH threshold.
**A positive trend in OS only for lung adenocarcinoma.

between both NGS and WES performed techniques ($p < 0.001$). Notably, the effect of TMB as a biomarker was predictive rather than prognostic. In this study, no correlation was found between high TMB and survival in those patients who were not treated with CPI.

Data from part 1 of Checkmate-227 were recently presented [37]. The study randomised patients with untreated advanced NSCLC to receive nivolumab plus ipilimumab (NIVO/IPI) versus nivolumab versus platinum-doublet chemotherapy. One coprimary endpoint of part 1 was PFS (assessed by blinded independent central review) with NIVO/IPI versus chemotherapy in a subpopulation of patients with TMB $\geq$ 10 mut/Mb determined by the FoundationOne CDx assay. Of the 1739 randomly assigned patients, 1004 (57.7%) had valid data for TMB-based efficacy analyses. Notably, about 44% of the patients had at least 10 mut/Mb, including 139 patients assigned to NIVO/IPI and 160 patients assigned to chemotherapy. In the population of patients whose TMB could be evaluated, there was no correlation between the TMB and PD-L1 expression level. For patients with a high TMB ($\geq$10 mut/Mb), there was a longer median PFS (7.2 versus 5.5 months, HR 0.58; 97.5% CI, 0.41 to 0.81; $P < 0.001$) with NIVO/IPI than with chemotherapy. There was no difference in median PFS between NIVO/IPI and chemotherapy in the low TMB (<10 mutations per megabase) population.

Other studies evaluating the role of TMB in NSCLC are summarised in Table 2 [38].

Invariably, TMB alone may not be enough to best predict response to CPI in NSCLC. Important correlations between high TMB and increased tumour-infiltrating lymphocytes (TILs), expression of proinflammatory cytokines and immune-related genes have been observed; the impact on clinical outcome has yet to be elucidated [39,28].

2.1.2. Mismatch repair and DNA replications genes

DNA mismatch repair (MMR) is a system for recognising and repairing mutations that can arise during DNA replication and recombination. Microsatellite instability (MSI) is the condition of genetic hypermutability and represents the phenotypic results of MMR deficiency (dMMR). While homologue recombination (HoR) genes (such as BRCA 1/2) are widely used to accurately repair harmful breaks on strands of DNA, known as double-strand breaks. Finally, polymerase ε (POLE) is a gene implicated in the replication of DNA [40]. Mutations occurring in all these genes can lead to increased genomic instability, and their alterations can accurately predict increased TMB and high neoantigen load [41,42]. In general, NSCLC has a low high-MSI incidence (approximately 0.6%) [43]. In a recent analysis from 5895 lung cancer tumours, only 0.3% were MSI-H and 1% of patients had mutation in DNA repair gene (MLH1, MSH2 and POLE), and most MSI-H patients (30/31) had high TMB ($p < 0.0001$) [28]. In tumours with dMMR, a high TMB and neoantigen burden is associated with favourable response to CPI [22]. Although MMR and MSI-H have established prognostic implications in colorectal and endometrial cancer, its role in lung cancer is less certain [39]. In their article, Rizvi et al.[21] identified three deleterious mutation alterations: POLE, POLD1 and MSH2 in three NSCLC responder patients to pembrolizumab with high TMB. In particular, one of the patients with POLD1 E374K mutation was never smoker with DCB whose tumour harboured the higher number of non-synonymous mutation load. Finally, updated data from Le et al.[44] reported an ORR of 53% and 64% 2-year survival rate in dMMR or MSI-H tumours treated with pembrolizumab in a range of different cancer types; however, no patient with NSCLC was included in this study. Response to nivolumab, ipilimumab and durvalumab in dMMR or MSI-H tumours has also been reported [45–47]. Based on these findings, FDA granted the first tissue/site-agnostic approval to anti–PD-L1 (pembrolizumab) for MSI-H and dMMR progressive advanced solid tumours.

2.1.3. Tumour microenvironment

The tumour microenvironment (TME) is a composite of immune cell infiltrate, fibroblasts, vascular and lymphatic endothelial cells in addition to signalling molecules. Cancer cells promote immune escape by modifying their surrounding environment to promote tumour proliferation and survival [48,49]. Within the TME, a diverse population of recruited immune host cells exists. These include mediators of the adaptive immune system; T and B lymphocytes, dendritic cells and effectors of the innate immunity; macrophages, polymorphonuclear (PMN) leukocytes and natural killer cells [48,49]. These immune cell infiltrates represent the host's attempt to interfere with tumour proliferation through immune surveillance and editing [50]. The immune contexture [51], a term describing the functional orientation, location and density of immune cell infiltrate, is prognostic of patient outcomes in multiple tumour types and bears potential value in predicting response to immune checkpoint blockade with high density of infiltrate associated with improved response to checkpoint blockade in so-called immunologically 'hot' tumours.

The prognostic impact of TILs has been reported in colorectal cancer [52–54], melanoma [55,56] and triple-negative breast [57,58] cancer with high densities of T cells (CD3+), cytotoxic T cells (CD8+) and memory T cells (CD45RO+) correlating with improved disease-free survival and OS. In NSCLC, a meta-analysis evaluating 7006 patients with stage I-IV NSCLC reported that CD8+ T cell infiltrate was the best predictor of survival [59]. In this study, high levels of CD8+ TILs conferred with a good prognostic effect on OS (HR 0.91,

p = 0.013) and recurrence (HR 0.74, p = 0.001). High FoxP3+ regulatory TIL levels were associated with poorer survival (HR 1.69, p = 0.042) and recurrence (HR 1.79, p = 0.001) [59]. In another series of 797 cases of patients with resected stage I-III NSCLC, stromal $CD8^+$ TIL was an independent prognostic factor survival and within each pathological stage [60].

Densities of CD3+ and cytotoxic CD8+ T cells in the tumour and in the invasive margin can be used to calculate the Immunoscore® in colorectal cancer, a validated scoring system with prognostic relevance [61]. In a similar system to the Immunoscore®, future validation of TILs in NSCLC could incorporate evaluation of stromal CD8+ TIL density and location to assess risk and guide adjuvant treatment strategies. It should be noted, however, that most of the data on the prognostic impact of TILs come from analysis of resected specimens where the TIL density and location can be readily observed. This has overwhelming limitation in advanced NSCLC as a prognostic tool, given the paucity of resected tissue in these patients. Furthermore, a differential TIL composition may be observed between primary and metastatic sites. In a study of 73 NSCLC patients with matched primary tumour and brain metastasis specimens, the infiltration density of CD3+ (p = 0.002) and CD8+ (p = 0.003) significantly correlated between primary and cerebral metastatic tumours; however increased CD3+, CD8+, CD45R0+ and PD-1+ TILs were more frequently observed within the primary tumour [62]. Novel methods such as multiplex immunofluorescence [63,64], and digital analysis software [65], may better characterise TIL subsets over traditional IHC; however, will require prospective validation.

Limited data are available on evaluating TILs as a predictive biomarker of checkpoint blockade in NSCLC. The Baseline tumour PD-L1 and immune cell expression (including CD3+, CD4+ and CD8+ lymphocytes) was assessed with IHC and prospectively analysed in 65 advanced NSCLC treated with nivolumab [66]. Stromal expression of PD-L1, CD3+, CD4+ and CD8+ immune cells predicted response to nivolumab. In 98 patients with advanced NSCLC treated with nivolumab, the percentage of stromal TILs was evaluated with hematoxylin and eosin (H&E) staining of archival tissue. The median TIL density was 5% (range 2–15%). The TIL density of ≥5% correlated with PFS on multivariate analysis (HR 0.31, CI 0.14–0.68 p = 0.004) and higher objective response (OR = 3.5, 95% CI [1.06–11.7], p = 0.04) [67]. The presence of both CD8+ T cells and PD-L1 was predictive of response to durvalumab in advanced NSCLC compared with expression of either CD8+ or PD-L1 alone [68]. Improved OS was observed in patients with CD8+/PD-L1+ tumours (24.3 months, CI 14.5–NE) compared with CD8+ (17.8 months, CI 14.0–NE) or PD-L1+ (17.1 months, CI 9.8–25.3) alone. Although exploratory data, these are encouraging results towards an improved predictive biomarker or composite biomarker system. Prospective evaluation of DNA and protein biomarkers will be assessed in a phase II study evaluating ipilimumab plus nivolumab in previously untreated advanced NSCLC with ORR as a primary endpoint (NCT03425331).

2.1.4. Immune gene signatures

Gene expression profiling uses reverse transcriptase polymerase chain reaction or DNA microarrays to simultaneously determine the expression of thousands of genes. Multiple gene signatures appear prognostic in NSCLC [69]. Interest has turned to identify immune gene signatures as prognostic and predictive markers. A meta-analysis of 18000 tumours over 39 cancers identified that expression of *KLRB1* (killer cell lectin-like receptor subfamily B, member 1) on multiple T-cell subsets was prognostic of improved survival in multivariate analysis (HR 1.5; 95% CI 1.3–1.8, p < 0.0001) [70]. From a data set of 5295 breast, colon, lung, ovarian and prostate tumours, nine individual immune-enriched gene signatures were identified, and all of them were prognostic in one or more tumour types (p = <0.05) [71]. Most identified immune gene signatures derived predominantly from infiltrated immune cells.

Immune gene signatures have demonstrated to be predictive of response to checkpoint inhibition in patients with melanoma treated with ipilimumab [72] and predictive of response in selected vaccine therapy trials [73]. Limited data inform the predictive role gene expression or immune gene signatures have in NSCLC treated with checkpoint blockade. Exploratory analysis from the phase II POPLAR [74] study, evaluating atezolizumab versus docetaxel in previously treated NSCLC, found that high T effector IFN-γ gene expression levels, an indicator of pre-existing immunity, were associated with improved survival in atezolizumab-treated patients.

2.1.5. IFN-γ related mRNA-based signatures

Upregulation of PD-L1 expression is mediated by IFN-γ. As a mechanism of immune escape and acquired resistance to PD-1 blockade, tumour cells interfere with the interferon-signalling pathway [75,76]. In a small study including 17 patients with NSCLC treated with nivolumab, archival tissue samples were examined for the effect of gene mRNA expression levels on survival and response [77]. A number of key genes were examined, including STAT3 and Rantes, YAP1 and CXCL5, DNMT1, RIG1 and TET1, EOMES, IFNG, PD-L1 and CTLA4, IKBKE and NFATC1. Gene expression levels were divided into high-expression and low-expression groups. IFNG mRNA expression, the gene encoding IFN-γ, emerged as the only biomarker with significant impact on treatment outcomes. High IFN-γ mRNA expression was associated with improved median PFS compared with low expression (5.1 versus 2 months, HR 6.66; 95% CI 1.2–36.79, p = 0.0297). No

significant difference in OS was found between the high and low IFN-γ mRNA expression groups (10.2 versus 4.9 months, HR 4.1, 95% CI 0.5–8.7), although arguably a clinically meaningful difference was achieved.

2.2. Biomarkers related to the host

Traditionally, most of the evidence around prediction of response to CPI has focussed on tumour characteristics; however, the host immune system plays a central role in driving response to immunotherapy. As elegantly showed in a preclinical model, a sustained immune cell proliferation in the periphery is required for effective tumour eradication. In addition, blocking the migration of immune cells from tumour to secondary lymphoid organs negatively affects the outcome to anti–PD-1 treatment [78]. The evaluation of peripheral blood (PB) immune cell counts and phenotypes and of the commensal gut microbiota as prognostic/predictive biomarkers to CPI in patients with advanced NSCLC currently represents an exciting area of discovery (Table 3).

2.2.1. Peripheral blood biomarkers: lymphocyte, neutrophil, eosinophil, monocyte and platelet counts

The assessment of the prognostic and predictive value of PB biomarkers in patients treated with CPI is attractive owing to the easy accessibility and limited costs of these parameters. In patients with nivolumab-treated NSCLC, the baseline absolute lymphocyte count (ALC) $\geq$ 1000/μL was significantly associated with PFS (HR = 0.55, p = 0.04) and OS (HR = 0.36, p = 0.03) (Table 3) [79]. However, at least one study showed that raised ALC levels were not predictive of OS benefit from ipilimumab [80], suggesting that although ALC may mirror the capacity of immune system to be activated by anti-CTLA4 or anti–PD-(L)-1 agents, it cannot adequately distinguish between immune suppressive and stimulatory interactions.

Different from ALC which indirectly measures the anticancer immune response, ANC, neutrophil-to-lymphocyte ratio (NLR) and derived NLR (dNLR) may reflect cancer-associated inflammation, a key determinant of disease progression and survival in several solid tumours [81].

Table 3
Host-related biomarkers in patients with advanced NSCLC treated with immunotherapy.

Biomarker	Patients No	Drug	End-point (HR and P value)	Comment
Baseline ALC≥ 1000/μL [79]	134	Nivolumab	OS (HR = 0.36, p = 0.03) PFS (HR = 0.55, p = 0.04)	Good OS Good PFS
Baseline ANC≥ 7500/μL [79]	134	Nivolumab	OS (HR = 3.46, p = 0.0) PFS (HR = 3.97, p = 0.001)	Poor OS Poor PFS
Baseline NLR ≥ 5 [88]	175	Nivolumab	OS (HR = 2.07, p = 0.002) PFS (HR = 1.43, p = 0.04) ORR (OR = 0.89, p = 0.75)	Poor OS Poor PFS
Baseline log (NLR) [89]	52	Nivolumab	OS (HR = 5.01, p < 0.001) PFS (HR = 2.09, p = 0.007) RR 40% versus 20% versus 0% p = 0.03	Poor OS Poor PFS Poor response
6 weeks NLR≥ 5 [90]	54	Nivolumab, pembrolizumab	OS (HR = 3.82, p = 0.003) PFS (HR = 15.09, p < 0.001)	Poor OS Poor PFS
Baseline dNLR > 3 [94]	466	PD-(L)-1 inhibitors	OS (HR = 1.70, p < 0.001)	Poor OS
6 weeks PLR ≥ 169 [90]	54	Nivolumab or pembrolizumab	OS (HR = 1.56, p = 0.02) PFS (HR = 1.80, p = 0.08*)	Poor OS
Baseline log (PLR) [89]	52	Nivolumab	OS (HR = 3.32, p < 0.001) PFS (HR = 1.38, p = 0.21*) RR 36% versus 20% versus 6% (p = 0.10)	Poor OS
Baseline AEC ≥ 150/μL [79]	134	Nivolumab	OS (HR = 0.24, p = 0.003) PFS (HR = 0.53, p = 0.02)	Poor OS Poor PFS
HLA-I Homozygosity [126]	NA¤	PD-(L)-1 inhibitors	OS (HR = 1.38, p = 0.003†) (HR = 1.60, p = 0.05§)	Poor OS
Akkermansia muciniphila (microbiome) [138]	60	PD-1 inhibitors	ORR 69% of PR, 58% of SD, 34% of PD (p = 0.007)	Good response

NSCLC, non–small-cell lung cancer; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; NLR, neutrophil-to-lymphocyte ratio; dNLR, absolute neutrophil count/(white blood cell count–absolute neutrophil count); PLR, platelet-to-lymphocyte ratio; AEC, absolute eosinophil count; HLA-I, human leucocyte antigen class I; PD-(L)-1, programmed death (ligand)-1; NA, not available; OS, overall survival; PFS, progression-free survival; ORR, overall response rate; HR, hazard ratio; OR, odds ratio; PR, RECIST partial response; SD, RECIST stable disease; PD, RECIST progressive disease; RECIST, Response Evaluation Criteria in Solid Tumours.
*Univariate analysis.
¤Overall population 1535 patients (different tumour types).
†Germline.
§Somatic.

Neutrophil infiltrate dominates the immune cell composition in NSCLC and inversely correlates with $CD8^+$ and $CD4^+$ immune cells in the TME [82]. Interestingly, in a proinflammatory status, an 'emergency granulopoiesis' with rapid release of immature or poorly differentiated neutrophils has been described in association with tumour progression [83]. In addition, *in vivo* models demonstrated that high tumour associated neutrophils correlated with resistance to PD-1 blockade, whereas neutrophil-depleting agents were able to reverse this phenomenon [84].

The baseline ANC≥ 7500/μL correlated with worse OS (HR = 3.46, p = 0.03) and PFS (HR = 3.97, p = 0.001) in patients with advanced NSCLC treated with nivolumab (Table 3) [79]. Besides ANC, several studies explored the negative prognostic value of high NLR. A meta-analysis reported NLR>5 as a poor prognostic factor in 3656 patients with advanced NSCLC [85]; in addition, 3.19 [86] and 3 [87] cut-off values of NLR were significantly associated with worse PFS and OS in two retrospective cohorts of advanced NSCLC patients treated with first-line platinum-based chemotherapy. In patients with advanced NSCLC treated with nivolumab, worse OS and PFS significantly correlated with a pretreatment NLR≥ 5 (HR OS = 2.07, p = 0.002, HR PFS = 1.43, p = 0.04) [88], and NLR was measured as a continuous variable (HR OS = 2.09, p = 0.007, HR PFS = 2.09, p = 0.007) (Table 3) [89]. In addition, 6 weeks after treatment, NLR≥ 5 was associated with poor PFS (HR = 15.09, p < 0.001) and OS (HR = 3.82, p = 0.003) in anti-PD-1–treated advanced NSCLC (Table 3), and the dynamic monitoring of the ratio between before and after anti–PD-1 treatment NLR significantly correlated with PFS (6.2 months for ratio before and after NLR≥1 versus 3.0 months for ratio <1, p = 0.035) [90].

dNLR (defined as absolute neutrophil count/[white blood cell count–absolute neutrophil count]) may be more relevant than NLR because it includes monocytes and other granulocyte subpopulations. High dNLR correlated with shorter OS in several tumour types [91–93]. In patients with advanced NSCLC treated with anti–PDL-1 agents, the baseline dNLR >3 was associated with worse OS (HR = 1.70, p < 0.001) and together with lactate dehydrogenase (LDH), composed the lung immune prognostic index, a parameter which efficiently classified patients into three prognostic groups with different survival outcomes (mOS: 16.5 versus 10 versus 4.8 months, p < 0.01) (Table 1) [94].

Finally, absolute eosinophil count (AEC), absolute monocytic count (AMC) and platelet-to-lymphocyte ratio (PLR) recently emerged as compelling biomarkers of outcomes to CPIs. The baseline AEC ≥150/μL was significantly associated with OS (HR = 0.24, p = 0.003) and PFS (HR = 0.53, p = 0.02) also in patients with nivolumab-treated advanced NSCLC (Table 3) [77]. Although AMC≥ 650/μL negatively impact OS (HR = 2.2, p = 0.001) in patients with ipilimumab-treated melanoma [95], currently, no evidence is available regarding the interplay between monocytes count and survival outcomes in patients with ICB-treated NSCLC.

However, lymphocyte-to-monocyte ratio ≥3.68 correlated with worse OS in 1453 patients with advanced NSCLC, confirming the potential negative prognostic significance of circulating monocytes [96]. Finally, in patients with advanced NSCLC, significantly longer OS to nivolumab treatment was associated with both elevated baseline PLR (HR = 3.32, p = 0.001) [84] and 6-week PLR ≥ 169 (HR = 1.56, p = 0.002) (Table 3) [90].

2.2.2. Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a very heterogeneous population with potent immunosuppressive properties [97]. It is possible that the main role of MDSCs is the protection of the host from excessive inflammation-related tissue damage during cancer or infections. Human MDSCs express markers of myeloid lineage, as CD33 and CD11b, and are an admixture of monocytic and granulocytic subpopulations [97]. PMN MDSCs are HLA-DR-CD14-$CD15^+$ or $CD66b^+$, whereas monocytic (M) MDSC are HLA-DR $^{-/low}$ $CD14^+$ CD15-[98].

No specific markers currently allow a clear discrimination between conventional CD15 + neutrophils and PMN-MDSCs in humans [98]. Because PMN-MDSCs seem to have different density compared with conventional CD15 + neutrophils, most studies identified PMN-MDSCs as $CD15^+$ cells remaining in PB mononuclear cell (PBMCs) layer during Ficoll gradient separation. However, recent data suggest that depending on methods applied and post-Ficoll manipulation of $CD15^+$ cells, human conventional CD15 + neutrophils also can be present in PBMC layer during Ficoll gradient separation, and, as PMN-MDSCs, these $CD15^+$ cells are able to impair T-cell proliferation *in vitro* [99].

In patients with advanced NSCLC, the frequency of peripheral M-MDSCs was significantly associated with AMC, and the increased level of M-MDSCs correlated with worse PFS to chemotherapy treatment (3 versus 9 months, p < 0.01) [100]. Similarly, in patients with NSCLC, peripheral M-MDSCs ($CD11b^+$ $CD14^+$ $CD15^-$) expressing the additional myeloid marker S100A9 [101] and tumour-infiltrating B7-H3–positive M-MDSCs [102] negatively correlated with PFS to chemotherapy (9.2 versus 3 months, p < 0.01) and postsurgical recurrence-free survival, respectively. Remarkably, in patients with NSCLC, the baseline frequency of a peculiar $CD15^+$ subset of M-MDSCs ($CD14^+$ $HLA\text{-}DR^-$ Lin^- $CD33^+CD11b^+$) had a detrimental effect on PFS (HR = 2.4, p = 0.002) to chemotherapy and OS (HR = 2.3, p = 0.008) [103].

Furthermore, an increase in $CD15^+$ M-MDSCs during treatment compared with the baseline was also observed in patients with NSCLC progressing to chemotherapy [104].

As for M-MDSCs, also the baseline levels and on treatment, elevation of circulating PMN-MDSCs correlated with lower levels of peripheral $CD8^+$ T-lymphocytes and decreased responsiveness to chemotherapy in patients with NSCLC [105]. In addition, PMN-MDSCs significantly decreased after three cycles of bevacizumab-containing regimens, suggesting a possible interplay between MDSCs and tumour-related angiogenesis [104].

In most of the aforementioned studies, several MDSC-related immunosuppressive mechanisms, such as the induction of nitric oxide synthase [101,105], arginase 1 [101,105] or IL-13/IL-4Rα axis [101], the production of reactive oxygen species [103], the increase of T-regulatory cells (T-regs) and IL-10 levels [102], the direct inhibition of T-lymphocyte proliferation and IFN-γ production [100,101] and the expression of tolerogenic biomarkers [106], were reported.

Few studies have explored the role of MDSCs in ICB-treated cancer patients. The baseline circulating M-MDSCs significantly correlated with response to ipilimumab in patients with advanced melanoma [107]. In addition, the frequency of peripheral MDSCs ($Lin^-HLA\text{-}DR^-CD33^+CD11b^+$) decreased more in patients responding to tremelimumab + IFN-α compared with non-responders [108]; similarly the M-MDSCs rate increased after the first ipilimumab dose compared with the baseline in patients with non-responding advanced melanoma [109]. Two other studies showed that lower frequencies of M-MDSCs correlated with longer OS, both at the baseline (2-year OS = 34.5% versus 0%, $p < 0.001$) [95] and at weeks 6 (HR = 0.38, $p < 0.005$) [110] after ipilimumab initiation (cut-off values of 5.1% and 14.9%, respectively).

To date, no study has explored the predictive/prognostic value of MDSCs in patients with anti-PD-(L)-1–treated advanced NSCLC. A comprehensive immunophenotyping of MDSCs and of circulating neutrophils and monocytes is of paramount importance in the CPI era. In fact, peripheral neutrophil and monocytic counts (ANC, NLR, dNLR and AMC) do not specifically distinguish all the heterogeneous myeloid cells involved in cancer-related inflammation and progression. For example, although AMC negatively predicted OS to ipilimumab treatment in melanoma, elevated levels of both classical ($CD14^+CD16^-$) $HLA\text{-}DR^{high}$ [111] and non-classical ($CD14^+CD16^+$) $HLA\text{-}DR^+$ [112] circulating monocytes recently correlated with higher responses to anti–PD-1 agents and ipilimumab, respectively, in patients with advanced melanoma. Therefore, it is likely that immature ($HLA\text{-}DR^-$) poorly differentiated populations increase in proinflammatory conditions [113], and they share common features of MDSCs, being highly immunosuppressive and functionally different from mature neutrophils or $HLA\text{-}DR^+$ monocytes. These 'early-stage' MDSCs [98] may be included in ANC, NLR, dNLR and AMC, potentially explaining the poor survival outcome associated with these blood parameters.

2.2.3. Lactate dehydrogenase

Cancer cells consume high levels of glucose resulting in increased lactate production even under aerobic conditions to meet the demands of rapid growth and proliferation. This process, termed the Warburg effect is catalysed by the metabolic enzyme LDH [114]. Elevated serum LDH levels are associated with worse prognosis in many solid tumours (e.g. melanoma), including lung cancer [115]. Different data at the low baseline LDH level reported favourable OS in patients with melanoma treated with anti–PD-1 agents [116–118]. A recent meta-analysis including 4084 patients with lung cancer demonstrated that higher pretreatment LDH levels were significantly associated with an increased risk of overall mortality in patients with lung cancer (HR = 1.49, 95% CI, 1.38–1.59) [119]. Serial LDH levels were evaluated in 94 NSCLC patients treated with anti–PD-1/PD-L1 agents. The baseline LDH level of less than 400 was associated with improved OS compared with patients with the baseline LDH >400 (NR versus 8.23 months, HR 0.45 95% CI 0.24–0.84) [120]. Elevated baseline LDH (defined as LDH > ULN) may also predict for early death (p0.036; OR 1/44.6) in patients treated with second-line CPI [121]. Finally, LDH was incorporated together with NLR in the lung immune prognostic index [89]. Larger studies and prospective analysis are warranted to define the prognostic impact of elevated LDH on NSCLC treated with CPI and its potential predictive role.

2.2.4. Regulation of immune-related genes and single nucleotide polymorphisms

The downregulation of HLA-I expression is a frequent mechanism of immune escape in various human malignancies [122–124]. The major genes whose structural alteration affects HLA-I expression are those encoding HLA-I heavy chains, β2M and components of the antigen-presenting machinery. Heterogeneity of HLA-I expression has been associated with different stages of tumour development. In the early phase (phase I, 'permissive'), cancer cells are HLA-I positive, and TILs are able to recognise and eliminate them; in the late phase of tumour development (phase II, 'not permissive/encapsulated'), HLA-I downregulation on tumour cells allows them to avoid the TIL attack, and HLA-I–negative tumour cells are encapsulated by an immune suppressive stroma consisting of MDSCs, T-regs and M2 macrophages [122]. In NSCLC, HLA-I loss was associated with increased PD-L1 staining on immune cells and elevated subclonal mutations [125], a

known poor predictive factor of immune checkpoint blockade [34]. Furthermore, HLA-I loss was found in 40% of early-stage NSCLC and was enriched in metastatic disease, suggesting a positive selection for this immune evasive mechanism during the natural history of the disease [125]. Conflicting results have been reported for the predictive role of HLA-I in cancer patients treated with PD-1/PD-L1 inhibitors. Some studies on melanoma suggested a significant higher HLA-I expression in responders compared with non-responding patients both in pretreated samples [126] and during the course of anti–PD-1 therapy [127]; however, these data were not confirmed in other series [128,129]. Of note, although HLA-I loss due to β2M truncation was reported as a mechanism of acquired resistance in one patient with advanced melanoma on PD-1 blockade [76], up to now, evidence regarding the role of HLA-I loss in patients with NSCLC treated with anti–PD-1/PD-L1 agents is missing.

2.2.5. Microbiome

Increasing evidence suggests a key role of the intestinal microbiota in shaping response to anticancer treatments. Two *in vivo* studies showed that an intact commensal microbiota is critical for therapeutic efficacy of oxaliplatin-based [130] or cyclophosphamide-based [131] chemotherapies. Mechanistically, the gut microbes enhanced chemotherapy-induced anticancer response by increasing ROS production from tumour-infiltrating myeloid cells [130] or by generating bacteria-specific T-helper (Th) 17 [131] or Th-1 [132] responses. Interestingly, the level of *Enterococcus hirae* and *Barnesiella intestinihominis* memory Th-1 cells correlated with improved PFS to platinum-based chemotherapy in 38 patients with advanced lung cancer [132]. The first demonstration of the role of microbiota in modulating responsiveness to immune checkpoint blockade was reported for CTLA4 inhibitors. Fecal microbiota transplantation (FMT) of different enterotypes from patients with advanced melanoma to mice recipients revealed that *Bacteroides* species (i.e. *B. thetaiotaomicron* and *B. fragilis*) induced a microbiota-specific T-helper 1 response and were associated with enhanced clinical benefit to anti-CTLA agents [133]. However, conflicting evidence showed that a high proportion of *Bacteroides* was present at the baseline in patients with poor outcomes to ipilimumab, whereas *Faecalibacterium* species were mainly enriched in responding melanoma patients [134]. This discrepancy may mirror a potential low concordance between the phenotype of the microbial composition between mice recipients and human donors, as reported also in other experimental settings [135]. Regarding anti–PD-(L)-1 agents, both a preclinical model [126] and a study using FMT [135] showed that *Bifidobacterium* species (i.e. *Bifidobacterium longum*) and some other microbes (i.e. *Collinsella aerofaciens*, *Enterococcus faecium*) enhanced antigen-specific tumour-infiltrating CD8+ T cells and correlated with improved response to PD-(L)-1 inhibitors [136]. In line to what observed in ipilimumab-treated patients, also in patients with melanoma receiving anti–PD-1 agents, *Bacteroides* species were enriched in non-responders; on the contrary, high alpha diversity and abundance of *Faecalibacterium* species were associated with increased levels of circulating effector T cells, decreased frequencies of peripheral T-regs and M-MDSCs and response to anti-PD-1 therapy [137]. Up to now, only one study explored the role of microbiome in patients with anti-PD-1–treated NSCLC. In 100 patients with cancer (including 60 NSCLC) *Akkermansia muciniphila* was significantly enriched in responders compared with progressing patients (69% versus 34% $p = 0.007$) and correlated with enhanced Th-1 cytokine (i.e. IL-12) production and increased intratumoral CD4/Foxp3 ratio [138]. In conclusion, comparison between differentially enriched commensal microbiota should be addressed with caution because of the existing diversity of experimental settings and to the potential bias related to the reconstitution of patients' microbiome phenotype in mice models.

2.3. Circulating T cells, PD-1 and PD-L1 and liquid biopsy

In the era of precision medicine, the concept of non-invasive sample collection to monitor response dynamics and predict resistance remains very attractive. Peripheral blood sampling, aka 'liquid biopsy' to detect T790M resistance mutations in EGFR mutant NSCLC is already in routine use. The limiting use of this method in CPI-treated patients is that the immune profile of PB cells differs greatly from that of solid tumours [139,140]. Soluble PD-L1 has been investigated as biomarker of response in melanoma, with increasing levels in response to CPI. High PD-1, PD-L1 and PD-L2 expression on PBMCs in 70 patients with NSCLC and 10 healthy subjects using a multiparametric flow cytometry demonstrated a worse OS, suggesting a tumour escape mechanism and a negative prognostic role of PD-1 and its ligand in PB [141]. A change in the number level of CD-8 T cells in PB after anti–PD-1 correlates with response in NSCLC [142]. A small series evaluating patients with NSCLC treated in the neo-adjuvant setting demonstrated an increased frequency of T-reg after ipilimumab [143]. Other recent studies confirmed the observation that the early clonal peripheral T-cell expansion correlate with response to CPI in NSCLC, and TCR expansion could be useful in measuring treatment response and acquired therapeutic resistance through loss of neoantigens [144,145]. Retrospective analysis from POPLAR and OAK studies demonstrated that high blood TMB (bTMB), measured by Foundation Medicine's assay, is associated with longer PFS and OS to atezolizumab in 211/273 patients

from POPLAR (OS HR 0.56 and PFS HR 0.57 for ≥ 16 mut/Mb) and 583/797 from OAK (OS HR 0.64 and PFS HR 0.65 for ≥ 16 mut/Mb) study. This is the first exploratory analysis to demonstrate that measuring bTMB may predict benefit in the second-line NSCLC therapy. In addition, no correlation was found between PD-L1 expression in tissue and bTMB, [146]. Use of ctDNA to detect early treatment response was demonstrated in patients with melanoma with reduction of the ctDNA level at 12 weeks, a predictor of response to CPI [147]. A study evaluating 15 patients with different types of cancers, including NSCLC, receiving anti–PD-1/PD-L1 therapy, compared the baseline and week 8 ctDNA. A high correlation between the ctDNA level and tumour size was individualised [148]. Development of blood-based assays to monitor CPI response remains a rapidly evolving field. Finally, this assay will be integrated as a companion diagnostic, in the first phase III Blood First Assay Screening Trial (BFAST) which will evaluate bTMB as a non-invasive marker of response to atezolizumab as first-line treatment for patients with advanced NSCLC [149]. Also, blood circulating tumour cells were used to predict response to immunotherapy. A study has reported that circulating tumor cells (CTC) can be useful for evaluating the efficacy of NK cells therapy in patients with NSCLC [150,151]. Exosomes, extracellular vesicles released from immune and cancer cells, are also under investigation as potential biomarker of response to CPI. Recent data demonstrate that PD-L1 expression on circulating exosomes changes during treatment and correlates with positive response to CPI on melanoma patients [152].

3. Discussion and conclusions

Immune CPIs, particularly anti–PD-(L)1 drugs are now firmly embedded in the treatment algorithm for treatment-naive and pretreated advanced NSCLC patients. Furthermore, very recently, the anti–PD-L1 durvalumab has been FDA approved as a consolidation strategy for unselected (by the PD-L1 status) NSCLC patients treated with definitive concurrent chemoradiotherapy.

PD-L1 expression is the only biomarker, albeit imperfect, currently used in clinical practice to select patients most likely to benefit from this class of drugs. This field is fast paced, and recent data indicate that TMB may enter clinical practice as a biomarker to aid selection of treatment-naïve patients who are potential candidates for dual blockade with ipilimumab and nivolumab.

Several questions still remain unanswered: for example, how to best select patients more likely to derive a survival benefit from first-line chemotherapy-IO combinations or how to predict which patients should be treated with front-line single-agent anti-PD(L1), despite low/negative PD-L1 tumour expression. Realistically, there will not be a single answer for a multitude of clinical scenarios. Several phase III studies that aim to address these questions are ongoing, and it is likely that the treatment landscape will radically change over the next 3–5 years.

The research community should focus on rational combination of drugs, appropriate patients selection and clinically relevant and patient-centred outcomes. This is the only way to make progress, avoiding a high burden of unnecessary adverse events and financial toxicity for patients and health systems.

Conflicts of interest statement

Raffaele Califano received honoraria from BMS, AZ, Roche and MSD; Benjamin Besse received institutional grants for clinical and translational research AstraZeneca, BMS, Boehringer Ingelheim, Lilly, Pfizer, Roche-Genentech, Sanofi-Aventis, Servier, Onxeo, OncoMed, Inivata, OSE Pharma and Loxo. The other authors declare no conflicts interest.

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