Review Article

Predictive biomarkers of immunotherapy for non-small cell lung cancer: results from an Experts Panel Meeting of the Italian Association of Thoracic Oncology

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Abstract: Unleashing the potential of immune system to fight cancer has become one of the main promising treatment modalities for advanced non-small cell lung cancer (NSCLC). The knowledge of numerous factors that come into play in the cancer-immunity cycle provide a wide range of potential therapeutic targets, including monoclonal antibodies that inhibits the programmed death-1 (PD-1) checkpoint pathway. Over the last two years, nivolumab, pembrolizumab and atezolizumab received approval for treatment of pretreated advanced NSCLC, and more recently, immunotherapy with pembrolizumab is the new standard of care as first-line in patients with high levels of programmed death-ligand 1 (PD-L1) expression. Selection of patients is mandatory and PD-L1 is the only biomarker currently available in clinical practice. However, PD-L1 staining is an imperfect marker, whose negativity does not exclude a response to immunotherapy, as well as the roughly half of patients are "not-responders" despite high tumor PD-L1 levels. The right cut-off, the differences among various immune checkpoint inhibitors and among various antibody clones, and a not trivial activity reported even in PD-L1 negative tumors are questions still open. New biomarkers beyond to PD-L1 assays as well as new strategies, including combination of immune checkpoint inhibitors are under investigation.

Keywords: Checkpoint inhibitors; immunotherapy; programmed death-1 (PD-1); programmed death-ligand 1 (PD-L1) expression

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Introduction

Worldwide, lung cancer is the most prevalent form of cancer, and its non-small cell subtype (NSCLC) constitutes up to 85% of cases. It remains one of the most frequent causes of cancer deaths. Lung cancer is a heterogeneous disease, characterized by a variety of different biomarkers and histologies, whose knowledge is important for deciding the most appropriate therapy. For many years, the benefits achieved by chemotherapy in advanced lung cancer were relatively small, associated to a substantial toxicity. In the

last decade, the development of novel therapeutic agents targeting the epidermal growth factor receptor (EGFR) and the anaplastic lymphoma kinase (ALK), both more effective and better tolerated than chemotherapy in the "biomarker-selected" patients, have changed the clinical practice, making essential the biomarkers' testing before recommending personalized treatments. Firstly, sensitizing mutations of the EGFR and, secondly, rearrangements of *ALK* gene have been validated as biomarkers that predict response to specific classes of drugs. Despite the clinical activity observed with drugs targeting EGFR or ALK, tumors eventually acquire resistance and overall survival (OS), even if improved as compared to platinum-based chemotherapy, remains poor. Furthermore, excepting for Asian population, less than 20% of lung cancers carry one of these two alterations, and the continue search for new molecular markers and for drugs that can be used to treat the remaining majority of patients is mandatory.

Unleashing the potential of immune system to fight cancer has become one of the main promising treatment modalities. The huge number of genetic and epigenetic changes occurring into cancer cells provide a diverse set of tumor-associated antigens that the host immune system can recognize, thereby requiring tumors to develop specific immune resistance mechanisms. An important mechanism of immune resistance involves immune-inhibitory pathways, called immune checkpoints, which normally mediate immune tolerance and mitigate collateral tissue damage. Two immune checkpoint receptors most actively studied in the context of clinical cancer immunotherapy are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4; also known as CD152) and programmed cell death protein 1 (PD-1; also known as CD279). As inhibitory receptors, they regulate immune responses at different levels and by different mechanisms. Playing in the priming phase (occurring in lymph node), CTLA4 is a potent co-inhibitor involved in early T-lymphocyte tolerance: it is expressed exclusively on T-cells, where it primarily regulates the amplitude of the early T-cell activation. In the subsequent effector phase, PD-1 expression is induced on activated T-cells, limiting their activity in peripheral tissues and the autoimmunity. After a successful immune response leading to antigen elimination, the PD-1 expression declines; otherwise, prolonged antigen stimulation leads to elevated PD-1 expression and is associated with an "exhausted" T-cell phenotype. Notably, PD-1 is more broadly expressed than CTLA-4, and it can be induced on other activated non-T lymphocyte subsets, such as B cells and natural killer (NK) cells, limiting their lytic activity (1). The interaction between PD-1 and its ligands programmed death ligand 1 (PD-L1; also known as B7-H1) and PD-L2 (B7-DC) reduces T-lymphocyte function triggering an intracellular dephosphorylating pathway, that leads to the cell death (reduced T-cell receptor signaling, reduced cytokine production, reduced T-cell cytolysis, altered lymphocyte motility, metabolic reprogramming) (2).

The cancer-immunity cycle is initiated by the release of antigens from dying tumor cells, which are taken up by antigen-presenting cells (APCs), such as dendritic cells (DCs). Following antigen uptake, DCs migrate to the draining lymph node to present processed tumor-associated peptides in the context of major histocompatibility complex class I (MHC-I) molecules to CD8+ T cells. The recruited T-cells traffic to and infiltrate into tumors, recognizing and finally killing the tumor cells (3). The numerous factors that come into play in the cancer-immunity cycle provide a wide range of potential therapeutic targets, and the consequent need of potential predictive biomarkers. Many tumors have increased expression of PD-L1 as important mechanism of immune evasion, suggesting PD-1/PD-L1 pathway blockade as a therapeutic strategy in cancer.

Over the last two years, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) have granted approval to the anti-PD-1 inhibitors, nivolumab and pembrolizumab, for the treatment of patients with advanced NSCLC with progression on or after first-line therapy, while to date, the anti-PD-L1 inhibitor atezolizumab is approved for the same indication only by FDA. Recently, both the European and U.S. agencies have extended the recommendations for pembrolizumab to the first-line therapy of PD-L1 "strongly positive" advanced NSCLC, reaching an important inflection point in the history of cancer therapy. In addition, other anti-PD-L1 agents, durvalumab and avelumab, are being investigated for the treatment of NSCLC. Predictive biomarkers that can direct the rational use of PD-1/PD-L1 checkpoint inhibitors are crucial given the risk of life-threatening immune-related complications associated with these therapies and the reality that most patients still do not benefit from their use. Furthermore, a biomarker-driven selection of immunotherapy responders and non-responders would reduce the financial burden for health systems due to these expensive treatments. The overexpression of PD-L1 by immunohistochemistry (IHC) have demonstrated to improve clinical outcomes in patients treated with anti-PD-1 and anti-PD-L1-directed therapy.

Clearly, PD-L1 staining is an imperfect marker, whose negativity does not exclude a response to immunotherapy, as well as the roughly half of patients are "not-responders" despite high tumor PD-L1 levels. The refinement of existing biomarkers and identification of novel predictive biomarkers will be key to ensuring the effective and safe use of these agents.

Methods

The Expert Panel Meeting on the PD-L1 role for immunotherapy in NSCLC patients was held on 3 March 2017 in Rome, Italy. Five medical oncologists, two pathologists and one clinical pharmacologist, each one from Italy, formed the scientific panel. Published data useful for panel discussion were identified by a PubMed search, performed with combinations of the following search terms: 'non-small cell lung', 'immunotherapy', 'PD-1' and 'PD-L1'. Only articles written in English were considered. For the discussion, each panelist selected the references that were considered relevant to the assigned topic. Abstracts presented between 2009 and 2017 at the main international meetings also were searched. Relevant references from selected articles also were included, and other articles were selected from the personal collections of the panelists. The level of evidence and the strength of recommendation have been evaluated according to Center for Disease Control and Prevention grading system (4).

The role of PD-L1 status for first-line immunotherapy

The anti PD-1 agents

Pembrolizumab is a type IgG4, non-killer isotype, humanized monoclonal antibody (moAb) against PD-1, with increased activity proportionally to expression of PD-L1 in tumor cells. According to results of phase III KEYNOTE-024 trial, pembrolizumab is set to become a new standard of care for first-line treatment of advanced NSCLC with high PD-L1 expression [defined as expression in at least 50% of tumor cells, tumor proportion score (TPS) ≥50%]. In this trial, the efficacy of pembrolizumab (at 200 mg every 3 weeks) compared to standard platinum-based chemotherapy was evaluated in 305 untreated patients with advanced NSCLC and with a specific molecular signature: PD-L1 TPS ≥50%, no activating mutation of EGFR or translocation of ALK. The primary endpoint of progression free survival (PFS) was significantly improved by approximately four months with immunotherapy [10.3 *vs.* 6.0 months, hazard ratio (HR): 0.50]: the hazard ratios favored pembrolizumab in all subgroups examined, with lower benefit in never smokers. After the superior efficacy of pembrolizumab at second interim analysis (at 6 months OS rate: 80.2% *vs.* 72.4%; HR: 0.60, P=0.005), the trial was stopped early by the external data and safety monitoring committee, with patients in the chemotherapy group given the opportunity to receive pembrolizumab. This significantly prolonged OS data was remarkable (70% alive at one year compared to 54% on chemotherapy), given that more than 40% of patients crossed over from the control arm to pembrolizumab after progression of the disease. Notably, responses to pembrolizumab treatment were higher (45% *vs.* 28%) and longer (not reached *vs.* 6.3 months), with the same time to response of chemotherapy responders (median time to response, TTR: 2.2 months) (5). These impressive data provide an opportunity for those patients without oncogenic alterations, whether squamous or non-squamous histology. Notably, this group of patients with strong positive PD-L1 expression seems not so poor, with a 30% of frequency reported in the trial (twice of approximately 15% of patients with oncogenic-addicted tumors). Pembrolizumab became the first immune checkpoint inhibitor to be approved by the FDA and EMA for first-line therapy in NSCLC patients with PD-L1 expression of 50% or greater. The KEYNOTE-042 trial (NCT02220894) is examining pembrolizumab in patients with NSCLC having 1% or more PD-L1 positivity in their tumors, instead the current threshold for clinical use of the 50% or more.

In contrast, the phase III CheckMate-026 trial failed to demonstrate a significant survival difference with nivolumab, another type IgG4, non-killer isotype, fully human moAb against PD-1, compared to platinum-based doublet in untreated patients with PD-L1 expression greater than 5% (median survival: 14.4 months with nivolumab *vs.* 13.2 months for chemotherapy, HR =1.02). Why nivolumab did not do similarly to pembrolizumab is unclear, but the main reason for this discrepancy may be the different patient selection of CheckMate 026 trial, enrolling the broader population with PD-L1 ≥1% (PD-L1 ≥5% for the primary analysis). Comparing baseline characteristics, the nivolumab group had fewer patients with PD-L1 expression ≥50% than chemotherapy (32.5% *vs.* 46.7%). If these imbalances would have made that a big difference in the large trial as CheckMate-026 is unlikely, but not

Table 1 Anti PD1/PD-L1 agents as first-line therapy: phase III trials with pending results

Trial	Histology (SQ or no-SQ)	Pts	Control arm	Experimental arm	PD-L1 status	Recruitment
Nivolumab						
Checkmate 227 (NCT02477826)	Both	1,980	PB-CT	Arm A: N alone; Arm B: N + I; Arm C: N+ CT	All comers	Yes
Pembrolizumab						
Keynote 042 (NCT02220894)	Both	1,240	SOC CT	Monotherapy	Positive (>1%)	No
Keynote 407 (NCT02775435)	SQ	560	CBDCA/Tax or CBDCA + Nab-P	Combined to CT of control arm	All comers	Yes
Keynote 189 (NCT02578680)	No-SQ	580	CDDP/CBDCA + Pem ×4 →Pem maintenance	Combined to CT of control arm as 1st line and maintenance	All comers	No
Durvalumab						
NEPTUNE (NCT02542293)	Both	960	SOC CT	Combined to tremelimumab	All comers	Yes
PEARL (NCT03003962)	Both	440	SOC CT	Monotherapy	Positive (≥25%)	Yes
MYSTIC (NCT02453282)	Both	675	SOC CT	Mono and combined to tremelimumab	All comers	No
Atezolizumab						
IMpower 110 (NCT02409342)	Both	570	CDDP/CBDCA + Pem or Gem	Monotherapy	Positive (TC 2/3 or IC 2/3)	Yes
IMpower 130 (NCT02367781)	No-SQ	650	CBDCA + Nab-P	Combined to CT of control arm	All comers	No
IMpower 131 (NCT02367794)	SQ	1,025	CBDCA + Tax/Nab-P	Combined to CT of control arm	All comers	No
IMpower 132 (NCT02657434)	No-SQ	568	CDDP/CBDCA + Pem ×4–6 → Pem maintenance	Combined to CT of control arm as 1st line and maintenance	All comers	No
IMpower 150 (NCT02366143)	No-SQ	1,200	CBDCA + Tax + Bev	Combined to CBDCA + Tax +/– Bev	All comers	No
Avelumab						
JAVELIN LUNG 100 (NCT02576574)	Both	1,095	CDDP/CBDCA + Pem	Monotherapy	Positive (≥1%)	Yes

Bev, bevacizumab; CBDCA, carboplatin; CDDP, cisplatin; CT, chemotherapy; Gem, gemcitabine I, ipilimumab; IC, immune cells; ICC, investigator's choice platinum-based chemotherapy; Nab-P, Nab-paclitaxel; N, nivolumab; Pem, pemetrexed; PB-CT, platinum-based chemotherapy, SOC, standard of care; SQ, squamous; Tax, Paclitaxel; TC, tumor cells. ClinicalTrials.gov.http://www.clinicaltrials.gov/. Accessed March 2017.

excluded. The post-hoc analyses based on various PD-L1 expression thresholds did not reveal notable differences in outcomes among the subgroup with 50% or more PD-L1 expression. Despite all potential bias of non-preplanned subgroup analysis, the response rate to first-line nivolumab among these patients who mirrored the KEYNOTE-024 population was lower than those to pembrolizumab (34% *vs.* 44.8%) (6).

Several trials evaluating strategy combinations as first-line setting are ongoing with aim to do even better and to achieve optimal therapeutic benefit also in patients affected by tumor with PD-L1 expression <50% (*Table 1*). Potential benefits of combining immunotherapy with chemotherapy are recently reported in KEYNOTE-021

phase II trial, where pembrolizumab given with standard first-line chemotherapy (carboplatin/pemetrexed) for untreated non-squamous NSCLC patients (any PD-L1, without targetable EGFR or ALK genetic aberrations) resulted in improved overall response rate (ORR: 55% *vs.* 29%, P=0.0016) and median PFS (13.0 *vs.* 8.9 months; HR: 0.53; P=0.0102) (7). Patients benefited regardless of PD-L1 expression, although those with the highest expression have higher response rates (ORR: 80% for PD-L1 ≥50%). The clear benefit in terms of PFS, exceeding 1 year for the first time, and the impressive response rate in the small group of patients with ≥50% expression, needed further explorations in the over international, randomized, double-blind, phase III KEYNOTE-189 study (NCT02578680) with pending results. In the squamous population, the KEYNOTE 407 trial (NCT0277543) is assessing the combination of pembrolizumab to chemotherapy (carboplatin/paclitaxel or carboplatin/nab-paclitaxel) as compared to chemotherapy alone. Another group of ongoing studies are assessing the role of the dual immune checkpoint blockade in the first line setting. In the phase I CheckMate 012 trial, the combination of the anti PD-1 nivolumab with the type-IgG1, killer isotype anti-CTLA-4 fully human moAb ipilimumab showed more efficacy, but higher overall toxicity, mitigated by different doses and schedules (delayed ipilimumab dosing, schedules with nivolumab at 3 mg/kg every 2 weeks. Recently, updated data for 129 patients from three of CheckMate 012's many cohorts were reported. With a median follow-up of 22 months in the monotherapy cohort (nivolumab at 3 mg/kg biweekly) and 16 months in the two combination therapy cohorts pooled (nivolumab at 3 mg/kg biweekly plus ipilimumab at 1 mg/kg every 6 or 12 weeks), the ORR were 23% and 43%, respectively. To note, the combination increased more than twice the response rates also in patients "refractory" to immunotherapy, such as never smoker (ORR: 27% *vs.* 9%) and EGFR-mutated patients (ORR: 50% *vs.* 14%) (8). The addition of ipilimumab resulted in longer PFS (median PFS: 8.0 *vs.* 3.6 months) and numerically higher 1-year OS rates (76% *vs.* 73% with combination and monotherapy, respectively), among patients unselected with respect to PD-L1 expression. Analyzing by PD-L1 status, the use of combination strategy in patients whose tumors did not exhibit PD-L1 expression (<1%) showed a modest activity, with little difference than nivolumab alone (18% for combination *vs.* 14% for nivolumab alone). However, the efficacy with nivolumab plus ipilimumab was enhanced with increasing tumor PD-L1 expression, reporting response rate of 92% in patients with ≥50% expression. Notably, the range of response rates reported in patients with at least 1% of positivity to PD-L1 and treated with combination strategies (57–92%) are superior to platinum-based first-line chemotherapy rates reported in literature. The PD-L1 testing still seems useful to predict also who could benefit from this new first-line approach. Although patient selection bias (presented combination therapy cohorts were enrolled after earlier cohorts with more frequent ipilimumab were poorly tolerated) and the small population of this phase I trial, the combination nivolumab 3 mg/kg be-weekly plus ipilimumab 1 mg/kg every 6 weeks is a future promising opportunity and it is being evaluated in further studies, including the phase III CheckMate 227 trial (NCT02477826).

The anti PD-L1 agents

Concerning anti-PD-L1 drugs, updated data from the phase II BIRCH trial showed long-lasting responses with atezolizumab, a type IgG1 fully humanized moAb, as first-line therapy for PD-L1 positive NSCLC patients, including only those with PD-L1 expression by IHC classified as TC2/3 (tumor cell membranes PD-L1 expression score of 2–3) and/or IC2/3 (immune cells membranes PD-L1 expression score of 2–3). Among the 138 patients of cohort 1, the ORR was 25% (18% with TC2/IC2 and 34% with TC3/IC3), with a median duration of response of 16.5 months. After a median follow up of 22.5 months, median OS with atezolizumab was 23.5 months (26.9 months with TC3/IC3), and the 12-month rate was 66.4% (61.5% with TC3/IC3) (9).

Ongoing phase III trials are comparing atezolizumab to chemotherapy in first-line setting, as single agent in the phase III IMpower110 trial (NCT02409342) enriched for PD-L1 expression, and as combination with chemotherapy in other trials (IMpower 130, IMpower 131, IMpower 132 and IMpower 150), each one enrolling for histologies and independently to PD-L1 status, but with subsequent pre-planned stratification for PD-L1 expression (*Table 1*).

Regarding durvalumab, a type IgG1 fully human moAb, as first-line monotherapy in advanced NSCLC, data from a phase I/II multicenter open-label study (NCT01693562), showed higher ORR in patients with high PD-L1 expression, staining in ≥25% of tumor cells (ORR: 25% *vs.* 6% for PD-L1+ and PD-L1–, respectively) (10).

In contrast, a phase Ib study combining durvalumab with tremelimumab, a IgG2 fully human anti-CTLA-4

moAb, in patients with advanced NSCLC (NCT02000947) demonstrated antitumor activity irrespective of PD-L1 status, including in patients without tumor cell membrane PD-L1 staining (11).

Based on these promising results, the MYSTIC (NCT02453282) trial are evaluating single agent durvalumab and the combination of durvalumab plus tremelimumab and the NEPTUNE trial (NCT02542293) durvalumab plus tremelimumab, respectively, as first-line in patients unselected for PD-L1 status, while the PEARL trial (NCT03003962) is evaluating durvalumab as single agent in PD-L1 positive patients (PD-L1 ≥25% tumor cells stained), each of three trials using standard of care treatments as comparator arms.

As first line, avelumab is another investigational anti-PD-L1 fully human IgG1-isotype moAb under evaluation in the large phase I multicohort dose-escalation and dose-expansion JAVELIN trial, enrolling over 1,700 patients with various tumor types. Preliminary findings for 156 NSCLC patients unselected for PD-L1 expression and treated with avelumab every 2 weeks as fist-line suggest a respectable activity, with an ORR of 22.4% after a minimum follow up of 13 weeks (12). The follow-up data are fairly immature at this point to suggest either superiority or inferiority in the efficacy over other checkpoint inhibitors. Currently, the JAVELIN Lung 100 phase III trial is ongoing, comparing single-agent avelumab with first-line chemotherapy in NSCLC patients with PL-1 expression at least of 1% (NCT02576574).

The role of PD-L1 status in second-line immunotherapy

Immunotherapy options for the second-line setting now include nivolumab, pembrolizumab (with PD-L1 expression broadened to include ≥1%), and atezolizumab, the third immune-check-point-inhibitor approved for previously treated patients.

First on scene, nivolumab is currently approved for both squamous and non-squamous advanced NSCLC across all PD-L1 expression levels, after survival data from two phase III trials, the CheckMate 017 and the CheckMate 057. These trials compared nivolumab every 2 weeks (3 mg/kg) to docetaxel every 3 weeks (75 mg/m^2) in patients with previously treated squamous (CheckMate 017) and non-squamous disease (CheckMate 057): the patients enrolled in both trials were unselected for PD-L1 status. In the phase III CheckMate 017 trial, involving 272 pretreated patients, nivolumab improved OS from 6.0 to 9.2 months (13). The survival benefit occurred irrespectively of PD-L1 expression for squamous histology, and was seen across all predefined subgroups (1%, 5%, and 10% as cutoff of PD-L1 expression level).

The primary endpoint of OS was met also in the non-squamous trial, reporting a significant 27% decrease in the risk of death (P=0.0015) and a long-term survival benefit, with half of patients alive at one-year (1-yr OS: 51% *vs.* 39% for docetaxel) (14). Survival data were recently updated at 2 years follow-up, and the fork across the curves remains: median OS was 12.2 months with the immune-checkpoint inhibitor and 9.5 months with chemotherapy (HR: 0.75; 2-yr OS: 29% *vs.* 16%) (15). Differing to squamous population, survival benefit of immunotherapy over docetaxel appeared to be linked to PD-L1 status. Using PD-L1 expression cutoffs as ≥1%, ≥5%, and ≥10%, median OS were substantially higher with nivolumab compared with docetaxel in all three subgroups, with the best survival improvement for patients with PD-L1 ≥10% (median OS: 19 *vs.* 8 months with nivolumab and docetaxel, respectively). The lack of difference between the two treatments in those patients with low PD-L1 levels (<1% or undetectable) suggests the PD-L1 expression as predictor of survival benefit for patients with non-squamous tumor (16). However, there is evidence that some patients with tumors lacking PD-L1 expression do benefit from nivolumab.

Notably, during the first 3 months of treatment survival in the overall population was poorer for nivolumab, with 15 excess deaths, primarily due to disease progression. In the attempt to identify which patients treated with nivolumab might be at risk of early death (occurring within 3 months of treatment), a multivariate analysis was conducted and recently presented: patients with any of the three features associated with poorer prognosis and/or more aggressive disease [fewer than 3 months since last treatment, progressive disease as the best response to the prior treatment, and Eastern Cooperative Oncology Group (ECOG) performance status of 1] added to lower or no PD-L1 expression appeared to be at higher risk of death on nivolumab than on docetaxel therapy (17). However the post-hoc, retrospective and unplanned nature of this analysis should be considered for data interpretation.

Regarding pembrolizumab, the open-label phase II/III KEYNOTE-010 trial showed a significant improvement in term of OS (one of co-primary endpoints) for both doses tested (at 2 mg/kg biweekly: HR 0.71, P=0.0008; at 10 mg/kg biweekly: HR 0.61, P<0.0001) compared

to docetaxel in patients with previously treated PD-L1-positive (≥1%) advanced NSCLC (18). No difference for PFS was reported in the total population, with a median time of 4 months for all three cohorts. The trial meets the other primary endpoints, improving both OS (at 2 mg/kg biweekly: HR 0.54, P=0.0002; at 10 mg/kg biweekly: HR 0.50, P<0.0001) and PFS (HR: 0.59 for both doses, P=0.0001 and P<0.0001 for 2 and 10 mg/kg, respectively) in patients with PD-L1 expression on ≥50% of tumor cells (18). These results highlighted the relationship between PD-L1 status and pembrolizumab efficacy beyond the first-line as well: the benefit increased with the proportion of tumor cells expressing the ligand, as previously reported in the large phase I KEYNOTE-001 (19). Updated data after one more year of follow-up confirmed the superiority of pembrolizumab to docetaxel: the median OS was 8.6 months with docetaxel, compared to 10.5 months (HR: 0.72) with lower-dose pembrolizumab and 13.4 months (HR: 0.60) with higher-dose pembrolizumab. Greater than 30% of patients with refractory lung cancer are surviving at 2 years with pembrolizumab, more than double that with chemotherapy (2-yr OS rate: 30.1% and 37.5% with pembrolizumab at 2 and 10 mg/kg, respectively, *vs.* 14.5% with docetaxel), with an apparent plateauing of the OS curves. Analysis restricted to the 47 patients who stopped pembrolizumab treatment after the two planned years showed responses in nearly 90%, with a durable clinical benefit (including stable disease). Responses are rapid (median TTR: 2 months), and durable (median DOR: not reached), with 72% still ongoing. Majority of patients who completed the entire treatment were initially responders (43 and 3 evaluable patients with PR and CR, respectively), despite the time to response ranged from 2 to 24 months. To note, 66% of these responding patients was strongly positive, compared with 42% of all patients on the trial given pembrolizumab. Despite the small numbers and the short follow-up, these data suggest that responding patients do not relapse early on planned cessation of pembrolizumab, and the "strong" PD-L1 expression seems to turn back again (20).

The antibody to PD-L1 atezolizumab (at recommended dose of 1,200 mg intravenously every 3 weeks) was recently added to the list of immunotherapy drugs approved by FDA in the second-line setting for NSCLC, regardless of tumor PD-L1 status, based on improved OS in two randomized clinical trials (OAK and POPLAR) comparing atezolizumab versus docetaxel in a total of 1,137 patients with NSCLC. After the gain in survival of 2.9 months achieved with atezolizumab in the phase II POPLAR trial, preliminary analysis of data from the phase III OAK trials confirmed a significant improvement in survival of the anti-PDL1 agent compared to docetaxel, in previously treated NSCLC patients, regardless of PD-L1 expression status or levels (median OS: 13.8 *vs.* 9.6 months in the atezolizumab and docetaxel arms, respectively; HR: 0.73; P=0.0003). Patients were stratified according to their level of PD-L1 expression on tumor cells or tumor-infiltrating immune cells (TC1/2/3 or IC1/2/3 population), and no statistically significant difference was found based on this expression: mortality was reduced in the highest PD-L1 stratum (≥50%) by 59%, but there was still a significant 25% improvement in survival also among those patients with negative PD-L1-expression (<1%) (21). Regarding histologic subgroup, patients with both squamous (HR: 0.73; P=0.0383) and non-squamous (HR: 0.73; P=0.0015) derived benefit from atezolizumab across PD-L1 expression levels.

Durvalumab demonstrated activity in heavily pretreated (≥3rd-line) advanced NSCLC patients that were enrolled in the phase II single-arm ATLANTIC trial (22). The study initially enrolled patients regardless the PD-L1 status, but was later restricted to patients with high expression of PD-L1 (at least 25% of tumor cells with membrane staining). The study included three independent cohorts, and results for the two cohorts having EGFR and ALK wild-type tumors were recently reported: the ORR was 16.4% and 30.9% in patients with ≥25% and ≥90% of tumor cells positive for PD-L1, respectively enrolled in cohort 2 and 3. Responses were also durable, with a median DOR not reached in patients of cohort 3 and more than 1 year in those highly positive of cohort 2. In term of survival, about half of patients with positive tumors were still alive at 1 year (1-yr OS rate: 47% for PD-L1 ≥25% and 50.8% for PD-L1 ≥90%). Consistently with other checkpoint inhibitors, the response rate to durvalumab as single agent rises proportionally with the tumor's level of positivity for PD-L1. The ongoing ARCTIC phase III study is investigating the safety and clinical activity of the durvalumab and tremelimumab combination as third line treatment of advanced NSCLC patients, regardless of PD-L1 status (NCT02352948).

The PD-L1 test: technical issues

The expression of PD-L1 by means of IHC represents the most validated predictor of response to anti-PD-1/PD-L1 inhibitors. Although it is still a matter of controversy, results

from clinical trials and recent pooled and meta-analyses have shown enhanced clinical benefit from anti PD-1/PD-L1 therapy in patients with PD-L1 positive NSCLC (23-26). The current one drug-one diagnostic test co-development approach for approval of therapeutic products in stratified or selected patient populations has resulted in each of the four therapeutic agents that are either FDA approved (nivolumab, pembrolizumab and atezolizumab) or in late-stage development (durvalumab) being associated with a unique anti-PD-L1 IHC assay. Two of the FDA approved PD-L1 IHC assays are manufactured by Dako, one using the 22C3 clone as a "companion diagnostic" for pembrolizumab (PD-L1 IHC 22C3 pharmDx), and one using the 28-8 clone as a "complementary diagnostic" associated with nivolumab (PD-L1 IHC 28-8 PharmDx). The other two assays (one approved and one not yet approved) have been developed with different detection technologies on the Ventana platform: the SP142 assay has been approved as "complementary diagnostic" for atezolizumab, while the SP263 clone is still under FDA regulatory process for treatment with durvalumab. Each of the four diagnostic assays is used to classify patients for treatment on the basis of measure of tumor cell membranes PD-L1 expression. Differing to others, the SP142 assay also includes a measure of infiltrating PD-L1-positive immune cells as part of the scoring guideline. A cut-off point is set for each test, and patients whose tumors score above the cut-off point are determined to be more likely to respond to the corresponding therapy (PD-L1 ≥1% for nivolumab; PD-L1 ≥50% and ≥1% for first and second line therapy with pembrolizumab, respectively; PD-L1 ≥25% for durvalumab; PD-L1 ≥50% as %TC or PD-L1 ≥10% as %IC, for atezolizumab).

To know whether the different assays identified the same patients, several comparative studies are tackling the issue of PD-L1 assay harmonization. Two multi-institutional efforts led by the National Comprehensive Cancer Network (NCCN/BMS PD-L1 partnership) and the IASLC (Blueprint study) are ongoing to assess the comparability and performance of different PD-L1 IHC assays in lung cancer. The results of the Blueprint PD-L1 IHC Assay Comparison Project indicate that there are both similarities and differences with respect to the four PD-L1 systems in terms of dynamic ranges, cell types stained, and overall staining characteristics. Three assays (22C3; 28-8; SP263) demonstrate similar analytical performance with respect to percentages of tumor cells positive and dynamic range, whereas the fourth (SP142) consistently labels fewer tumor cells. All of the assays detected immune cell staining, but with less precision in analytical performance than with tumor cell staining, probably due to higher pathologist variability when assessing inflammatory cell proportion score. By comparing and interchanging assays and cutoffs, slightly more than one-third of cases studied (36.9%) showed discrepant results for PD-L1 expression between the assays, leading to "misclassification" of PD-L1 status for some patients (27). Similar results were reported recently in a German comparison study, confirming the feasibility of a reproducible PD-L1 tumor cell scoring (28-8 and 22C3 assays yield comparable tumor cell percentages). Despite results are based on 15 cases scored by 9 pathologists, the choice assay may influence percentage of stained tumor cells (more stained with SP263, fewer with SP142) and intensity of stained immune cells (SP263 and SP142 stain more intense) (28).

The largest trials conducted by Ratcliffe and colleagues (approximately 500 tumor biopsy samples assessed) also confirmed a high analytical correlation between the three different commercially available diagnostic tests (Ventana SP263, Dako 22C3, Dako 28-8), achieving overall percentage agreement of more than 90%. The study showed that the patient population defined by Ventana SP263 at the 25% cutoff point is similar to the group identified by the Dako 28-8 at the 10% cutoff, and a high degree of concordance between the SP263 and 22C3 assays was demonstrated with 50% as cutoff point applied. These results suggest which cutoff points should be used to optimize agreement between a positive or negative PD-L1 result, and could be helpful to compare results from clinical studies that have used different tests (29). The use of the tests interchangeably is still too far, and further work, including independent review by other pathologists, will be needed to confirm the findings.

To date, pembrolizumab is the only PD-1/PD-L1 inhibitor that has an FDA and EMA indication restricted to PD-L1-positive tumors according to PD-L1 IHC 22C3 pharmDx assay labeled by FDA and CE-IVD. Recently, a 22C3 protocol designed for Ventana instrument showed highly concordant analytical results with the Dako PD-L1 IHC 22C3 PharmDx kit (30). Despite all limits, IHC test for PD-L1 remains the only validated procedure. In the next clinical practice, the use of PD-L1 tests will require a clarification of different pitfalls: the correct timing for PD-L1 test (if limited availability of tumor tissue, addiction or not of PD-L1 testing to other molecular studies, such as ALK FISH); their repayments, considering the

expensiveness; the cytological samples not yet validated; which test/platform has to be used; the correct report by pathologists, who must be trained in a specific program of formation before embarking in routine use of the tests.

The PD-L1 expression as predictive factor: open issues and new biomarkers

Despite the wide consensus on testing tumors for PD-L1 expression, as the current standard of care, this biomarker is limited by its "unperfected dichotomy" across studies and molecules: patients with low levels of PD-L1 expression have responded at rates of up to 17%, as well as the roughly half of patients are "not-responders" despite high tumor PD-L1 levels.

For evaluation of checkpoint inhibitors' effectiveness, PD-L1 is only one of three variables to consider: the immune system activation and the pair drug concentration-binding affinity play a key role as well as the expression of the pharmacological target PD-L1. The impressive clinical efficacy of these drugs in the melanoma, lung cancer and bladder cancer, known as immunogenic tumor types with higher somatic mutation prevalence, confirmed the role of cancer immunogenicity in the response to immunotherapy (31). A relationship between mutational landscape of lung cancer and sensitivity to PD-L1 blockade was recently suggested: higher non-synonymous mutation burden (HMB, as ≥200), leading to cancer-specific neoantigens, has been shown to correlate with a durable clinical benefit from pembrolizumab therapy in NSCLC patients, with an improvement of objective response and of PFS (HR: 0.19) (32). Notably, the molecular smoking signature (based on high transversion rates) correlated more significantly with nonsynonymous mutation burden than self-reported smoking history. Efficacy also correlated with specific DNA repair pathway mutations (genes encoding for RNA polymerase, anti-stress proteins, and other repair mechanisms are mainly involved). However, a number of responders carry a low mutation load, while non-responders may carry a high mutation load (32). These results highlight differences in the clinical relevance of different mutations and in mutagenic processes involved. On the other hand, another possible explanation is the degree of intratumor heterogeneity, in terms of even or uneven neo-antigen tissue distribution. Unlikely other studies, the heterogeneity of mutational load due to non-homogeneous tumor bulk was not evaluated in this study. The lack of validated cutoff (200 as arbitrary choice), the expensiveness of laboratory technique (whole-exome sequencing evaluated with next generation sequencing), the genomic variability of world population, limit the role of HMB as a routine clinical marker.

By targeting the patient's immune system, immunotherapies provide the potential to identify a uniform dose and schedule across multiple tumor types. Differing from cytotoxic drugs, the maximum tolerated dose (MTD) paradigm may be not the best approach for selection phase III doses of immuno-oncology agents, especially for well-tolerated agents, where MTD may not even be determined. Other factors can contribute to selecting a suboptimal biological dose: the typical 4-week observation period used to identify dose-limiting toxicities may not be sufficient for the delayed time of onset of immune-related AEs; the exposure-safety relationships are not yell well understood; dose selection for immuno-agents usually occurs in early-stage clinical trials and is typically based on tumor response (exposure-response relationships), but data are often limited by low number of patients and limited exposure durations, which may not fully represent the potential for improved OS (33).

During exposure-responses analyses, responders to nivolumab appeared to have higher drug exposures than non-responders at the same dose level, suggesting baseline clearance as potential predictor for drug's efficacy. Peripheral pharmacodynamic markers such as PD-1 receptor occupancy (RO) did not provide meaningful demarcation for dose selection (peripheral RO saturation at low exposures, corresponding to nivolumab dose of to 0.3 mg/kg biweekly). The utility of peripheral RO data is also hampered by its unknown relationships with intra-tumoral RO and with immune-modulating activity in the tumor microenvironment. No relationship between exposure and tumor shrinkage rate results, probably due to lymphocyte infiltrate or to lack of shrinkage in the early phase of treatment (34).

Finally, the same mechanism of action does not mean the same drug: nivolumab and pembrolizumab are two IgG4 moAbs targeting both PD-1, but differing for structure (fully human the first, humanized the second), and for binding affinity to PD-1, being the dissociation constant (Kd), an estimate of binding affinity, for pembrolizumab and nivolumab of 28 pM and 3 nM, respectively (Table 2) (2). If this difference in terms of binding affinity could explain different clinical outcomes is still unknown, considering the lack of validation for this parameter in early-stage clinical trials. The better knowledge of genetic variants of PD-1/

Table 2 Checkpoint inhibitors: differences of binding affinity

Drug	Target	Antibody type	Affinity/K2
Nivolumab	PD-1	Finally human IgG4	2.6 nM
Pembrolizumab	PD-1	Humanized IgG4 kappa	29 pM
AMP-224	PD-L2	PD-L2 IgG1 Fc fusion	NA
Pidilizumab (CT-011)	PD-1	Humanized IgG1 kappa	20 nM
Atezolizumab	PD-L1	Engineered IgG1 (no ADCC)	0.4 nM
Durvalumab	PD-L1	Modified IgG1 (no ADCC)	NA
Avelumab	PD-L1	Humanized IgG1	NA
BMS-936559	PD-L1	Humanized IgG4	NA

ADCC, antibody dependent cell-mediated cytotoxicity; Ig, immunoglobuline; NA, not available.

PD-L1 and their signal transduction pathways, the search of new markers of global immune system activation, and the role of drug-target affinity are open issues, yet. Regarding the global immune-responsiveness, current research is focusing on phenotypic markers, including blood levels of viruses highly diffused in the population, and on the patient's global immune-responsiveness, corresponding to selected laboratory parameters, such as the lymphocyte and eosinophil counts and the lymphocytes/neutrophils ratio. New biomarkers are being tested to understand who benefits more from immunotherapy. Tumors with "inflamed phenotype", featuring both high mutational load and extensive tumor T-cell inflammation, are theoretically the best responders to an anti PD-1 treatment (35). This could explain the lower responsiveness to immune checkpoint inhibitors of EGFR-mutated tumors, reporting high expression of PD-L1, but low mutational burden and less inflamed phenotype. However, the increment of mutational burden after acquiring resistance against EGFR-TKIs, could suggest the role of PD-L1 inhibitors in a delayed phase of EGFR-addicted tumors. The tumor T-cell inflammation, which correlates with CD8+ T-cell infiltration, gamma interferon expression, and indirectly PD-L1 and PD-L2 expression has been advanced as a potential biomarker to integrate PD-L1 status, above all the interferon inflammatory immune gene signature. The mutanome-directed immunotherapy is another promising way of future research, with the aim to bridge genomics and immunotherapies. Low intratumoral heterogeneity combined with high mutational/neoantigen load should correlate to best responsiveness to immunotherapy. In contrast, reduced responses to immunotherapy are related to the tumor aneuploidy, as somatic copy number alterations may represent markers of immune evasion. Also, the role of high microsatellite instability to predict immunotherapeutic response in NSCLC is still uncertain, differently to colorectal cancer. Next-generation DNA sequencing and targeted RNA sequencing—with the issue of several multiplexed gene panels to validate—protein analysis, immune-signature analysis with the simultaneous knowledge of various transcript by nanostring technologies, and liquid biopsy are proposing for evaluation of the molecular profiling in the cancer. Future research aims to obtain a cancer immunogram for each patient: the balance of several factors (PD-L1 status, intratumoral T-cells, lymphocyte count, mutational load, the absence of inhibitors such as interleukin-6, the inactivation of tumor metabolism, tumor sensitivity to immune effector interferon-γ sensitivity and MCH expression as biomarkers of tumor immune-sensitivity) makes every tumor both complex and unique. Recently, three immunogram patterns were described in lung cancer patients: T-cell-rich, T-cell-poor, and intermediate (36). However, these new approaches seem to be so far from clinical practice, technically difficult, too expensive, and not validated in pivotal trials. Furthermore, multifactorial markers may be more difficult to regulate and interpret clinically and that currently, no single biomarker can definitively exclude patients from therapy.

Conclusions

The immunotherapy still remains a way to find out, with issues to overcome and new targets to develop. The PD-L1 expression limits (spatial and temporal heterogeneity,

harmonization of the assays, the use of selection criteria for responder patients) needs to be clarified as soon as possible. Notably, not all patients will have sufficient tissue to test for PD-L1, and those untested patients will not have the possibility to get pembrolizumab as fist-line, but only nivolumab or atezolizumab in the second-line setting. Other factors concerning patient selection for pembrolizumab as first-line monotherapy are the exclusion of patients with (I) poor performance status; (II) untreated brain metastases; (III) preexisting autoimmune disorders; and (IV) EGFR mutant or ALK rearranged tumors, in addition to the tissue availability for PD-L1 staining and to the lesser percentage of strongly PD-L1 positive patients than reported in pivotal trial, will reduced about 50% of patients who could be treated. Among oncogenic addicted patients, prospective trials evaluating responsiveness to PD-1 inhibitors of those who develop resistance to targeted therapy are warranted. Finally, the time of treatment in clinical trials was until progression disease or, in the case of pembrolizumab trials for 2 years, and remains unknown if a brief course of a PD-1/PD-L1 inhibitor would be the same. Also the feasibility of retreatment for patients previously responders who went off therapy for various reasons, such as toxicities, should be evaluated. The light on all these points might avoid excess toxicity in patients, decrease the patient discomfort and not least would reducing the "financial toxicity".

Expert opinion: consensus for clinical practice

Does PD-L1 play a role as a target for immunotherapy?

For anti PD-1/PD-L1 checkpoint inhibitors, the role of PD-L1 is certain, representing, indirectly (anti-PD1 moAbs) or directly (anti-PD-L1 moAbs) their target. However, other factors might play a role in the different therapeutic activity, such as drug affinity for PD-1 or PD-L1, drug clearance, pharmacokinetic exposure of target tissues to the moAbs and global immune-responsiveness of the subject.

Is PD-L1 test reliable from a technical point of view?

Despite its limits, the PD-L1 test is robust, reproducible, and technically reliable on formalin-fixed paraffin-embedded samples, provided that companion-complementary diagnostic assays or laboratory validated tests are employed. Evaluation of PD-L1 on direct smears is not recommended. Further studies need to validate PD-L1 evaluation on cytological samples (cell blocks) and educational efforts are required to enhance scoring reproducibility among pathologists.

Is a "unique and harmonized" PD-L1 test possible and reliable?

This harmonization is recently confirmed as possible, using three assays with comparable characteristics and performances: Dako 22-8, Dako 22C3 and Ventana SP263. The fourth validated clone Ventana SP142 yields a weaker staining of tumor cells. High reproducibility can be reached in tumor expressing ≥50%, while in those expressing around 1% reproducibility can be lower.

Is PD-L1 test positivity "predictive" of higher efficacy for check-point inhibitors therapy in pretreated NSCLC patients?

Objective responses rates have been observed regardless PD-L1 positivity, despite a proportional correlation to PD-L1 expression (Tumor Proportion Score). Higher survival benefit, compared to docetaxel, was observed in highly PD-L1 positive NSCLC, except for nivolumab in squamous histology. Testing PD-L1 status in pretreated patients can be performed on primary or metastatic site in archive or re-biopsy samples.

Is PD-L1 test "predictive" of efficacy for check-point inhibitors therapy as first-line in advanced NSCLC?

Compared to standard treatment, survival benefit was observed only for pembrolizumab in NSCLC with high PD-L1 expression (≥50%). The PD-L1 expression should be tested upfront at time of diagnosis. Tumor sampling and tissue management should be improved to accommodate the need to test PD-L1 in squamous and non-squamous histology.

New biomarkers beyond PD-L1?

Emerging evidences suggest that PD-L1 expression is not the only determinant to predict immunotherapy clinical response. The use of multiple biomarkers may be foreseen in the future. Current investigation is addressing putative novel biomarkers, such as tumor mutation burden and immune signature on tissue and plasma, but nothing ready

Table 3 Consensus for translational research

Priority	Issues
High	Diagnostic reliability of PD-L1 tests on cytological samples and small biopsies
	PD-L1 levels on liquid biopsy
	Biomarkers of tumor foreignness
	Mechanisms of resistance in PD-L1 positive tumors
	Mechanisms of responsiveness in PD-L1 negative tumors
Moderate	Development of common strategies to detect at the RNA level gene fusion (ALK, ROS1, RET) and immune signature biomarkers
	Archival versus rebiopsy specimens for PD-L1 expression
	Heterogeneity of PD-L1 expression in primary versus metastatic sites
Low	Influence of prior treatments on PD-L1 expression
	Evaluation of the role of PD-L1 expression in NSCLC versus other solid tumors
	Correlation between PD-L1 expression and other driver mutations

PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer.

for clinical practice is yet.

Consensus for translational research (see Table 3)

Future research and development will focus on several issues, as contained in the *Table 3*.

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Footnote

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