Accepted Article

The path to a better biomarker: application of a risk management framework for the implementation of PD-L1 and TILs as immuno-oncology biomarkers into breast cancer clinical trials and daily practice

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Short running title: Implementation of PD-L1 and TILs as immuno-oncology biomarkers for breast cancer.

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Abstract

Immune checkpoint inhibitor therapies targeting PD-1/PD-L1 are now standard of care in oncology across several hematologic and solid tumor types, including triple negative breast cancer (TNBC). Patients with metastatic or locally advanced TNBC with PD-L1 expression on immune cells occupying ≥1% of tumor area demonstrated survival benefit with the addition of atezolizumab to nab-paclitaxel. However, concerns regarding variability between immunohistochemical PD-L1 assay performance and inter-reader reproducibility have been raised. High tumor-infiltrating lymphocytes (TILs) have also been associated with response to PD-1/PD-L1 inhibitors in patients with breast cancer. TILs can be easily assessed on hematoxylin and eosin stained slides and have shown reliable inter-reader reproducibility. As an established prognostic factor in early stage TNBC, TILs are soon anticipated to be reported in daily practice in many pathology laboratories worldwide. Since TILs and PD-L1 are parts of an immunological spectrum in breast cancer, we propose the systematic implementation of combined PD-L1 and TIL analyses as a more comprehensive immune-oncological biomarker for patient selection for PD-1/PD-L1 inhibition-based therapy in patients with breast cancer. Although practical and regulatory considerations differ by jurisdiction, the pathology community has the responsibility to patients to implement assays that lead to optimal patient selection. We propose herewith a risk-management framework that may help mitigate the risks of suboptimal patient selection for immuno-therapeutic approaches in clinical trials and daily practice based on combined TILs/PD-L1 assessment in breast cancer.

Keywords: PD-L1, TILs, breast cancer, biomarker risk-management, immunotherapy

Introduction

Immune checkpoint inhibitor (ICI) therapies targeting programmed cell death 1 (PD-1) and programmed death ligand 1 (PD-L1) are now standard of care in oncology. Anti-PD-1 pembrolizumab (Keytruda®, Merck & Co. Inc., Kenilworth, NJ, USA) and nivolumab (Opdivo®, Bristol-Myers Squibb Company, New York, NY, USA), and anti-PD-L1 atezolizumab (Tecentriq®, Genentech Inc, South San Francisco, CA, USA), durvalumab (Imfinzi®, AstraZeneca plc, Cambridge, UK) and avelumab (Bavencio®, Merck KGA, Darmstadt, Germany) have been approved to treat multiple tumor types, in many countries. To date, atezolizumab specifically has been approved for triple negative breast cancer (TNBC). At the same time, immunohistochemistry (IHC) based detection of PD-L1 expression has been proposed as the predictive biomarker to select patients that may benefit from these therapies. Five primary antibody clones have been developed in the form of assays paired with a specific staining platform. PD-L1 22C3 (Agilent Technologies Inc., Santa Clara, CA, USA), 28-8 (Agilent Technologies Inc.), SP142 (Roche Tissue Diagnostics, Tucson, AZ, USA), SP263 (Roche Tissue Diagnostics) and 73-10 (Agilent Technologies Inc.) have been used in clinical trials of the above-mentioned drugs, respectively. In addition, laboratory-developed tests (LDTs) using any of the above-mentioned primary antibodies or the E1L3N clone with different staining platforms are in use in research and clinical scenarios. Parallel to the multiple assays, multiple scoring systems exist. Table 1 shows technical details and defines scoring methods used for each antibody. Furthermore, different cut-offs are used to define PD-L1 positivity for different tumor types, while for certain indications PD-L1 testing is not required for PD-1/PD-L1 inhibition-based therapy, from now on referred to as ICI.

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For several years the oncology and pathology communities have raised concerns about the reliability of IHC based detection of PD-L1 to appropriately select patients for ICI. To date, while PD-L1 is currently the only approved biomarker for these agents, it remains controversial given the complexities of its clinical use due to variability in assay performance of the PD-L1 IHC antibodies, spatial and temporal heterogeneity, absence of a unified scoring system and concerns about inter-reader reproducibility for scoring PD-L1 on immune cell (ICs). Due to these inconsistencies, some patients who could benefit might not receive treatment while others may be treated based on erroneous test results, exposing them to potential adverse side effects with no drug benefit. Additionally, since PD-1/PD-L1 interaction is only one of many factors that may determine the clinical response to immunotherapeutics, it is unlikely that a single biomarker will sufficiently predict clinical outcomes in response to ICI. The use of composite biomarkers can provide biologically relevant information on multiple factors that determine response. In a meta-analysis, combined biomarker approaches such as PD-L1 IHC and tumor mutational burden (TMB) and multiplex fluorescent IHC evaluating protein co-expression and spatial relationships, demonstrated an improved performance over PD-L1 or TMB alone [1]. As guardians of patient's samples, pathologists partnered with clinicians, industry and regulators must guide evidence-based inclusion of biomarkers in clinical trials and daily practice to ensure the best patient outcomes possible. Stromal tumor-infiltrating lymphocytes (TILs) have also been studied as a predictive biomarker of response to ICI for a variety of cancers including breast cancer (BC). TILs can be assessed on a simple hematoxylin and eosin (H&E) slide with reliable reproducibility among pathologists when they adhered to the standardized method [2, 3]. We propose PD-L1 and TILs as a more comprehensive composite biomarker.

A good biomarker should be analytically valid, robust, reproducible and clinically useful. To be incorporated into daily practice, it must also be affordable and accessible to pathologists in both academic and community-hospital practices worldwide [4]. In this review, we propose a systematic implementation of combined PD-L1 and TIL analysis as a comprehensive immune-oncological biomarker for patient selection for ICI in both clinical trials and daily practice. In support of this position, we will outline the evolution of PD-L1 and TILs as biomarkers, from the analytical and clinical validation phases through clinical implementation, review the challenges we have encountered and propose mitigation approaches within a risk-management framework as previously published [5]. The collective of available evidence anticipates enhancement of patient selection and safety by the systematic implementation of combined PD-L1 and TIL analysis.

Technical validation phase: analytical validity of PD-L1 immunohistochemistry

Biomarker development starts with an initial discovery in pre-clinical studies, which we will not cover in this review, followed by a validation phase in which the biomarker is adapted to clinically applicable assay platforms and subjected to analytical and clinical validation [6]. For PD-L1 IHC, analytical validity refers to the accuracy and consistency of the technique to detect the presence of PD-L1 protein. To be able to analyse the accuracy and consistency of the test we must first define the presence of PD-L1 protein. PD-L1 can be expressed on solid and hematologic tumor cells (TCs) and on ICs, including macrophages, dendritic cells, lymphocytes and granulocytes [7, 8]. PD-L1 is expressed in the cytoplasm and/or on the cell membrane. A PD-L1 positive (PD-L1+) TC has been defined as showing partial or complete membranous staining of any intensity [8-13]. Accompanying cytoplasmic staining is often observed but

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ignored in TC. On the other hand, a PD-L1+ IC is one that shows membranous or cytoplasmic staining of any intensity. Cytoplasmic staining may show a punctate or granular pattern, most commonly observed with SP142 [11, 12, 14]. IC can be observed in aggregates or as single cells dispersed in the intra-tumoral or peritumoral stroma as well as admixed with TC [8, 14].

Chromogenic IHC based detection of PD-L1 has been largely concordant with other methods to detect PD-L1 expression, such as immunofluorescence, mass spectrometry and RNA *in situ* hybridization [9, 15-18]. Each PD-L1 diagnostic kit has shown precision, reproducibility and robustness when standard operating procedures and optimization of conditions are followed [8, 14, 19-21]. Studies comparing PD-L1 assays performance on archival, routine clinical practice and clinical trial TNBC samples have shown discrepancies among SP142, SP263 and 22C3 assays. PD-L1 positivity defined as the proportion of tumor area occupied by PD-L1 positive immune cells (IC_A) $\geq$1% with SP142 showed between 20-38%, 10-35%, and 7-19% fewer number of PD-L1+ cases compared to SP263 $IC_A\geq$1% and 22C3 combined positive score (CPS) $\geq$1 and $IC_A\geq$1% respectively [22-26]. Prevalence with each assay is shown in Table 2. Similar findings were observed in previous multi-institutional studies on archival clinical non-small cell lung cancer (NSCLC) and urothelial carcinoma specimens, in which results between 22C3, 28-8, SP263, 73-3 and E1L3N assays were broadly comparable, while SP142 has shown lower PD-L1 expression on both TC and IC [9, 10, 12, 13, 16, 38-43].

To investigate this discordance, a study mapped the antibody binding sites for each antibody [44]. SP142, SP263 and E1L3N bind amino-acid residues in the cytoplasmic tail of PD-L1 [14, 44, 45], while 22C3 and 28-8 target the extracellular domain [44, 46]. 22C3 and 28-8 binding sites contain N-linked glycosylation sites, which may lead to variability in antigen retrieval. N-glycosylation may also affect binding efficacy of antibodies with cytoplasmic

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binding, differences between mass spectrometry and E1L3N IHC were reported on melanoma samples with high glycan modifications, suggesting that posttranslational modifications could interfere with recognition of binding sites [17]. SP142 and SP263 bind to the same epitope [44], hence the above described discordance between these assays may be due to differences in assay protocol leading to insufficient antibody saturation. The visualization and amplification methods have been shown to affect the extent and pattern of expression of PD-L1 on IC and TC [47], at least partly explaining the discordance among assays.

Inter-observer reproducibility represents a major challenge to the reliable assessment of any IHC assay; this is especially true for PD-L1. While inter-pathologist reproducibility for the assessment of PD-L1 on TC is high, concordance has been lower for IC evaluation across multiple tumor types [10, 13, 39], irrespective of the assay. Scoring IC is harder from a methodological standpoint. Identification of IC may be straightforward in some cases, but complex in others, especially when trying to differentiate between TC and intra-tumoral monocytic (macrophages/dendritic) cells, which cannot easily be distinguished on H&E. Additionally, the four kits reportedly show different IC staining patterns: 22C3, 28-8 and SP263 assays mainly stain macrophages and dendritic cells, whereas the SP142 assay, while staining a lower number of ICs, also identifies some lymphocyte-like cells [47]. Using SP142, the majority of non-neoplastic cells were CD68+, while 5% were CD8+ [48]. Two multi-institutional studies, including up to 19 pathologists show moderate agreement (interclass correlation coefficient (ICC) 0.560-0.805) between pathologists for SP142 assay on triple negative breast cancer (TNBC) samples [23, 25]. Pathologists were trained on the evaluation of PD-L1 IHC and were required to pass a proficiency test in one of these studies [23]. Agreement for other assays was slightly lower. Table 3 shows details of studies evaluating inter-observer reproducibility on BC

samples. Of interest, SP142 has been shown to have the highest concordance among readers for PD-L1 IC≥1% in studies including other tumor types [10-12], though the differences are not statistically significant. This may be because SP142 stains TC with lower prevalence, allowing the IC staining to be more easily identified.

Overall percent agreement (OPA) is the proportion of samples that are classified the same by all observers. The Food and Drug Administration summary of safety and effectiveness data showed an OPA of 91.1% with SP142, however, this study only included 3 pathologists [14]. In contrast, the study including 19 pathologists found an OPA of 41% with SP142. Recently, Reisenbichler *et al*. [25] showed a new method for analysis of OPA as a function of the number of observers. If there is high concordance, then the plot will plateau at a high OPA with a small number of observers. They showed a decrease in OPA for PD-L1 IC_A≥1% as the number of observers increased, reaching a plateau of 40% at 9 observers. Results of real-world training conducted by Roche demonstrated an OPA of 98% between 903 pathologists from 75 countries assessing 28 TNBC cases in a proficiency test, however the methodology for calculating OPA was not disclosed on the abstract [49]. On re-analysis of the National Comprehensive Cancer Network (NCCN) study with lung cancer samples, OPA between 13 pathologists increased from 0% with a three-category score to 18% using a two-category scale (IC≥1% and <1%), or even 67% if an outlier pathologist is excluded [38], showing that two categories are more reproducible. Moreover, low values, such as 1%, show lower inter-reader reproducibility [51].

Clinical validation phase: clinical validity and utility of PD-L1 IHC and TILs as predictive biomarkers of response to PD-1/PD-L1 inhibitors

Clinical validation refers to how reliably the biomarker correlates with response to ICI and divides the patient population into groups with divergent expected outcomes. Clinical utility is a measure of whether clinical use of a test improves clinical outcome and assists clinical decision making [52]. The gold standard for evaluating biomarker clinical utility is the outcome of prospective randomized trials which include biomarker evaluation in the study design, such that it is powered to specifically evaluate the benefit derived from the new drug according to biomarker status [52-54]. However, most randomized trials adopt a primary endpoint of drug efficacy and do not employ a biomarker design. Table 4 shows the characteristics and results of clinical trials utilizing PD-L1 IHC and TILs as predictive biomarkers of response to ICI in BC.

Patients with newly diagnosed metastatic or locally advanced PD-L1 $IC_A \geq 1\%$ TNBC demonstrated survival benefit with the addition of the PD-L1 inhibitor atezolizumab to nab-paclitaxel in the randomized phase III IMpassion130 trial in which all patients were prospectively tested for PD-L1 with SP142 [28]. Evaluation of progression-free (PFS) and overall survival (OS) in the PD-L1+ subgroup was one of the primary efficacy endpoints. Even though primary endpoint of OS for the intention-to-treat (ITT) population was not reached, and although pre-specified statistical testing hierarchy prevented further formal analysis, OS was improved within the PD-L1+ subgroup with the addition of atezolizumab [28, 63].

No improved outcome was observed for pre-treated metastatic TNBC patients with PD-1 inhibitor pembrolizumab as monotherapy or compared to chemotherapy (treatment per physician choice: vinorelbine, capecitabine or gemcitabine) in the ITT population nor PD-L1+ populations (PD-L1 combined positive score (CPS)≥1 or CSP≥10 with 22C3) on the randomized phase III KEYNOTE-119 study [31]. Large randomized trials with survival endpoints, like the aforementioned, are generally required to establish medical utility of a predictive biomarker.

Nevertheless, retrospective analysis of specimens collected from prospective trials may also establish biomarker clinical utility if appropriately designed and if archival tissue is available from enough patients to have adequate statistical power [64]. An exploratory analysis with a cut-off of CPS≥20 did show a longer benefit in OS with the addition of pembrolizumab to chemotherapy [31]. To further reliably establish clinical utility, these results should be validated in similar, but separate cohorts [64]. Likewise, response to pembrolizumab monotherapy or in combination with chemotherapy was independent of PD-L1 status (CPS≥1) on single arm phase II KEYNOTE-086 and KEYNOTE-150 trials respectively [33, 34]. Of note, patients participating in these studies were pre-treated. TNBC patients with PD-L1 IC≥1% and IC≥5% showed improved survival outcomes with nivolumab after induction treatment on the phase II TONIC trial [36].

For patients with metastatic trastuzumab-resistant HER2-positive (HER2+) BC, PD-L1 CPS≥1 was predictive of response to the pembrolizumab plus trastuzumab combination in the single arm phase II PANACEA trial [58]. Conversely, on the phase II randomized KATE-2 trial, although response was numerically higher in patients with PD-L1 IC_A≥1% tumors, no significant benefit was observed with the addition of atezolizumab to T-DM1 [59]. Notably, in an exploratory biomarker-analysis, the hazard ratio (HR) for OS was similar for PD-L1 as for TILs in this trial, suggesting that both predict benefit from the addition of atezolizumab to T-DM1.

In the neoadjuvant setting, increase in pathological complete response (pCR) rate observed with the addition of pembrolizumab to chemotherapy was independent of PD-L1 status (CPS≥1) on the randomized phase III KEYNOTE-552 trial [60]. Similarly, PD-L1 $IC_{IC\%}$≥1% not only failed to predict pCR after the addition of durvalumab to chemotherapy, but in fact, was

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predictive of response in the chemotherapy only arm on the phase II randomized GeparNuevo trial [37].

Exploratory analysis of randomized phase III KEYNOTE-119 trial showed that patients with TILs higher than the median (5%) had better OS in the pembrolizumab monotherapy arm but not in the chemotherapy arm [55]. TILs greater than the median were also shown to be predictive of response to single agent pembrolizumab regardless of PD-L1 status on retrospective biomarker analysis of the previously treated PD-L1 unselected cohort A of KEYNOTE-086 (median TILs 5%), but even more so within PD-L1+ treatment naïve cases on cohort B (median TILs 17.5%) [57]. Furthermore, patients with TNBC and HER2+ BC that responded to treatment with pembrolizumab alone and in combination with trastuzumab showed higher median TILs on the single arm phase II KEYNOTE-086 and PANACEA trials [57, 58] and on the TONIC phase II trial evaluating nivolumab after induction treatment [36].

In the neoadjuvant setting, baseline TILs evaluated as a continuous variable and stratified (<10%, 11-59%, ≥60%) were predictive of pCR in both the durvalumab plus chemotherapy and chemotherapy plus placebo arms of GeparNuevo [37]. Additionally, overall T cell density was associated with pCR in response to pembrolizumab in the randomized phase II I-SPY 2 trial [65].

It is important to keep in mind that TILs have also proven predictive of response to neoadjuvant chemotherapy (NAC) in patients with TNBC and HER2+ BC [66, 67] and strongly prognostic of outcome in patients with early TNBC treated with standard anthracycline-based adjuvant chemotherapy [68-70] on phase III and pooled trials. In addition, in early stage treatment naïve TNBC patients, high TIL-counts predict >98% 5-year survival, suggesting that the benefit of chemotherapy is probably very limited in this group [71, 72]. PD-L1 baseline expression has also been positively associated with response to anthracycline-based NAC in

hormone receptor positive BC [73] and TNBC [74]. However, both PD-L1 and TILs are predictive of response to monotherapy ICI, proving predictive capacity beyond chemotherapy treatment.

Clinical implementation: inclusion of PD-L1 and TILs in clinical trials.

Given the existing evidence, we propose systematic implementation of combined PD-L1 and TIL analyses as a comprehensive immune-oncological integral biomarker for patient selection for ICI in BC clinical trials. Since both have proven to be influential determinants of response to ICI, the use of both markers as stratification factors on randomized clinical trial designs could improve balance of baseline characteristics among arms. Trial design should include PD-L1 and TIL analyses in real time, pre-specifying the inclusion of both biomarkers in the protocol and ensuring well-powered biomarker clinical utility data that can be used for regulatory submissions of both TILs and PDL1 as markers of efficacy for immunotherapy. Additionally, new protocols can be written to conduct prospective-retrospective biomarker analysis on archival tissues from completed trials. All studies must be conducted and analyzed in a standardized manner per Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria [75, 76]. TILs should be scored as recommended by the International Immuno-oncology Biomarker Working Group (TIL-WG) [2, 3] as a continuous variable with clinically relevant cut-offs in mind. A recent publication demonstrated the feasibility of the application of a web-based TILs scoring platform to enable the use of TILs as a stratification factor in an immunotherapy clinical trial for TNBC within a risk-management framework [77]. This pilot study proposes a standardized workflow that can be used in future clinical trials.

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In BC, both PD-L1 and TILs have shown higher expression in primary tumors than in metastases [2, 24, 57]. Nonetheless, PD-L1 expression on either primary breast (HR PFS: 0.61[0.47-0.81]) or metastatic lesions (HR OS: 0.55[0.32-0.93]) were both predictive of response to atezolizumab and nab-paclitaxel combination [24]. Although the most recent sample may be more representative of the current immunologic status, evaluating all available samples on clinical trials would provide useful data to define the most appropriate time point for testing. Pre and on-treatment TILs have been associated with response to ICI [61, 62]. On-treatment biopsies could be included in protocols, since they may provide real-time information to help guide future treatment choices.

Furthermore, the existence of multiple scoring systems for PD-L1 assays precludes the harmonization of assays and complicates reproducibility of scoring among pathologists. A single scoring system would allow a more accurate and direct comparison among assays and simplify scoring, likely facilitating adoption into clinical practice. For BC patients, clinical benefit has been correlated with PD-L1 expression on IC [7, 27, 35, 36]. Moreover, PD-L1 expression on macrophages was associated with outcome in response to neoadjuvant durvalumab [78]. While PD-L1 expression on TCs with SP263 was predictive of response to durvalumab in the neoadjuvant setting [37], in the advanced setting, expression on TCs evaluated by SP142 [7, 24, 27], 22C3 [36] and 73-10 [35] was not predictive. We therefore encourage reporting PD-L1 expression as IC, $TC_{\%}$/tumor positive score (TPS) and CPS separately for all assays in clinical trials to assess which scoring system is most clinically relevant for each setting. Note that IC scored as proportion of tumor area occupied by PD-L1 expressing IC is not equivalent to IC as a percent of TC, given that most BC contain distinct stromal areas in-between tumor areas; a score

normalized by cross-sectional area produces lower scores than a score normalized by number of TCs.

We believe that the application of systematic criteria for combined PD-L1 and TIL analyses to future clinical trial designs will produce reliable data to better understand which patients will benefit the most from ICI. The resultant data could ultimately allow the conduction of a meta-analysis to provide clinically impactful data. Nevertheless, PD-L1 expression and IC presence are subject to dynamic regulation processes that are biologically incompletely understood. Additionally, several other factors also influence responses to ICI, including tumor neoantigen load, IC composition, and expression of other costimulatory and inhibitory molecules. Additional biomarkers may help further refine patient selection. These potential biomarkers will likely be predictive in a tumor type specific dependent manner. For instance, TMB has been showed to be a predictive biomarker of response to ICI across multiple cancers in retrospective studies [79]. However, mutational load is relatively low in BC. Additionally, in TMB estimates are variable across laboratories [80], with slower turnaround and higher cost compared to IHC. HLA-DR tumor expression has been associated with response to ICI in breast [81] and other tumor types. Further investigation of these and other biomarkers in correlative studies in clinical trials is warranted, such as those evaluated by multiplex fluorescence IHC or gene expression profiling.

Clinical implementation: inclusion of PD-L1 and TILs in daily practice.

An analytically and clinically validated biomarker assay can be implemented into clinical care, but level 1 evidence is needed to change clinical practice. Results from randomized phase III

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IMpassion130 [28] led to the accelerated approval of atezolizumab and nab-paclitaxel as the standard treatment regimen for PD-L1+ ($IC_A \geq 1\%$) metastatic TNBC in many countries. Clinical implementation of a biomarker requires three key elements: regulatory approval, reimbursement by health systems and incorporation into clinical practice guidelines [6]. Regulatory approval is different in every country. Only the SP142 assay has been approved by regulatory agencies as the companion diagnostic test for the administration of atezolizumab and nab-paclitaxel in countries such as the United States of America (USA), Japan, Sweden, Peru and Argentina. Whereas in certain counties in the European Union (EU), China and Brazil, any PD-L1 assay can be used as long as it has been validated. In the EU, drugs are generally not regulatorily linked to a companion diagnostic test. The NCCN and other guidelines [82] include PD-L1 diagnostic testing as part of the workup for recurrent or metastatic TNBC as well as other tumor types. However, to date, in most countries, PD-L1 testing is not performed routinely on metastatic TNBC, but mainly upon oncologist request.

Following regulatory approval and incorporation into clinical practice guidelines, a biomarker must also be affordable and accessible to pathologists in both academic and community-hospital practices worldwide to be successfully incorporated into daily practice. In Japan, where the SP142 assay is the approved companion diagnostic test for TNBC, only this assay is covered by the health system. In the USA, the SP142 assay and LDTs are covered by health insurance. In Peru, PD-L1 testing is covered by prepaid health insurance but it is not yet covered by the public health system. In Argentina, Australia, Brazil, Chile, India, Morocco and some countries in the EU, the test is not yet covered by the health system. In the UK, the National Institute for Health and Care Excellence (NICE), the UK regulatory agency that evaluates drug efficacy, reported: "Atezolizumab with nab-paclitaxel [...] does not meet NICE's

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criteria for inclusion in the Cancer Drugs Fund. This is because it does not have the potential to be cost effective at the current price, and there is no clear evidence that further trial data would resolve the uncertainties" [83].

Subsequently, each pathology laboratory faces challenges including sample selection, sample processing, choice of assay, quality assurance, and interpretation to ensure correct implementation and consequent accurate patient selection. Table 5 summarizes these and previously stated risks along with proposed mitigation approaches to ease the implementation of PD-L1 testing into clinical practice. It has been suggested that labs should test as many time points as are available such as to maximize patient eligibility for treatment. However, such an approach will be costly without proven benefit to the patient. It is also unclear whether insurance companies will pay for testing of multiple samples.

From a clinical perspective, it is imperative that an assay identifies patients likely to respond to ICI, rather than identifying a greater proportion of PD-L1+ patients. The lower prevalence of PD-L1+ cases detected by the SP142 assay could potentially lead to fewer patients selected for therapy (false negative tests), while use of SP263 or 22C3 could lead to greater patient eligibility at the expense of false positive tests, unnecessarily subjecting a subset of these patients to toxicity and financial costs without clinical benefit. In an exploratory *post hoc* analysis of IMpassion130, the PD-L1+ population identified by each assay independently showed clinical benefit with similar HR (HR [95% CI]: SP142 $IC_A \geq 1\%$: PFS:0.60 [0.47-0.78], OS: 0.74 [0.54-1.101]), 22C3 CPS$\geq$1: PFS: 0.68 [0.56-0.82], OS: 0.78 [0.62-0.99], SP263 IC$\geq$1%: PFS: 0.64 [0.53-0.79], OS: 0.75 [0.59-0.96]) [24]. 22C3 and SP263 identified a larger PD-L1+ population, of which the SP142 positive cases are a subgroup. Of note, the biomarker evaluable population (BEP) only included 68% of the original ITT population, and while it may

be adequately sized to reliably identify a larger treatment effect in the two-category test positive patients, it could be underpowered to analyse a tripartite population of dual assay analysis. OPA for analytical concordance with SP142 ($IC_A \geq 1\%$) were 64% (22C3 CPS$\geq$1) and 69% (SP263 IC$\geq$1%), demonstrating that the assays are not equivalent [24]. Nevertheless, even if mostly driven by the SP142 positive subpopulation, SP263 and 22C3 identified patients that showed improved PFS and OS, making them clinically interchangeable since they identify populations with near similar clinical outcomes [9]. Further studies such as this, done in partnership between academia, industry and regulatory entities, need to be encouraged, preferably before formal regulatory approval of an assay as a companion diagnostic linked to a specific drug. In a meta-analysis including samples from various tumor types, each diagnostic kit was found to better match with properly validated corresponding LDTs than to other diagnostic kit assays [43]. Although further studies are warranted, the use of LDTs is a reality in daily practice.

From a practical point of view, a single pathology laboratory cannot have all assays available. Labs performing PD-L1 IHC testing for NSCLC already use other assays, most commonly 22C3 and SP263 assays or an LDT [38, 40]. Developing and validating the SP142 assay could be an unwarranted burden for some laboratories. SP142 and 22C3 commercial diagnostic assays are performed on different platforms, each a large capital expenditure. In countries where regulatory agencies permit, PD-L1 could be performed as an LDT, if analytically validated. For the SP142 antibody, similar PD-L1 expression was observed with different platforms [15], although using a different detection method has proven to impact assay performance [47]. In countries where the regulatory agencies mandate the use of the SP142 assay, smaller hospitals will likely need to outsource testing to a reference laboratory. To date, in most countries, only a handful of large academic hospitals and reference labs are performing PD-

L1 testing for TNBC. The choice of assay should be an agreement between pathologists, oncologist and patients, and be directed by good laboratory practices and common sense. Patient advocates need to be aware of how the choice of an assay can influence treatment decisions.

For quality assurance purposes, tonsil control tissue must be included as positive and negative controls alongside the clinical case to accept or reject the assay run. Tonsil tissue is recommended since it demonstrates granular punctate staining on lymphocytes arranged in aggregates and dispersed single cell patterns, diffuse staining in the reticulated crypt epithelium, and absence of staining on superficial squamous epithelium [8]. A control sample staining close to the cut-off point is also recommended [84]. Unlike HER2, PD-L1 has no reflex alternative testing method that can be employed to ascertain accuracy. Additionally, since the different PD-L1 assays are not equivalent, they cannot be tested against each other for accuracy. Pathology laboratories must audit their PD-L1 positivity rates as part of internal quality assurance. Prevalence of PD-L1+ (IC_A>1%) TNBC with SP142 was 41% (44% on primary and 36% on metastatic samples) on IMpassion130 [24, 28]. Other studies have shown a similar range of prevalence 32-58% on TNBC samples using SP142 IC_A≥1% [14, 22-25, 28-30]; one study had an outlier prevalence of 78%, in which the first 25 patients were selected only if PD-L1+, then enrolment was extended to all patients [7]. However, PD-L1+ prevalence reaches 54-87% and 46-86% when using SP263 IC_A≥1% and 22C3 CPS≥1 respectively [22-25, 31-34]. Prevalence of PD-L1+ on each of the cited studies is shown on table 2. As part of an external quality assessment and validation, samples with known PD-L1 expression should be tested and compared on proficiency tests. A validated standardized PD-L1 Index Tissue Microarray [16] containing cell line samples with known varying PD-L1 expression levels could be used for this purpose. For LDTs, laboratories must show results comparable to those obtained in clinical trials

with a diagnostic assay validated to predict potential response to a particular drug in a particular disease as a gold standard [85]. The Canadian Association of Pathologists has published a guide to ensure the quality of PD-L1 testing [86].

As previously discussed, inter-observer reproducibility is one of the main pitfalls regarding PD-L1 validity as a viable prognostic or predictive marker. These errors in patient selection not only put patients at risk, but also generate extra costs for health systems, generating issues at the national regulatory level regarding reimbursement-criteria. Pathologists must be trained to interpret and score PD-L1 assays. Training material developed by assay manufactures, including a digital training platform with a proficiency test, can be accessed freely [87, 88]. The value of training should be established in statistically rigorous studies that include post-training evaluation with proper decay time. Additionally, pathologists must participate in external quality assurance programs. A guideline for the interpretation of PD-L1 IHC developed by pathologists for pathologists, like those for TILs [2, 3, 89], ER [90] and HER2 [91], is needed. Such a guideline developed by the International Association for the Study of Lung Cancer is available [92]. Even though reproducibility among pathologists has been shown to be higher with a two-category scoring [38], we believe the percentage of PD-L1+ IC_A should be incorporated into the pathology report in addition to a positive or negative PD-L1 deliberation.

Another tool available for pathologists that can improve reproducibility is digital image analysis of whole-slide images. Evaluation of TILs in solid tumors is a highly suitable application for computational assessment, automated quantification by computer-based image analysis provides accurate and reproducible results that can aid pathologists, especially for borderline cases surrounding the clinically relevant 1% cut-off that are challenging to distinguish by eye. In the basic retrospective research realm, image analysis algorithms have shown better or

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comparable concordance between the automated algorithm score and the mean pathologist score than between pathologists [9, 93]. Like any biomarker, computer-based image analysis algorithms would need to be analytically and clinically validated with demonstrated clinical utility such that results are consistent with trial materials used to established cut points for clinical decision-making and approved by corresponding regulatory agencies before they can be implanted into daily practice. A recent publication outlines possible workflows and challenges for analytical and clinical validation of computational TILs assessment [94], paving the path for its incorporation into clinical trials and daily practice.

In view of the considerable level Ib evidence for the prognostic value of TILs, the expert panels at St Gallen 2019 [95] and authors of the 2019 edition of the *World Health Organization Classification of Tumors of the Breast* recommended quantification of TILs in TNBC. Internationally, some institutions have already begun incorporating TILs into pathology reports, paving the way for TIL counts to inform BC therapies. Going forward, a standardized format for reporting TIL counts, similar to those used to report hormone receptors, will need to be adopted. Given the inherent variability in TIL distribution and heterogeneity of sampling, we propose that TIL counts should be scored in treatment naïve and advanced setting BC specimens, while in the clinical post-treatment setting TILs should be scored only on clinical trial samples according to established guidelines [96]. TILs should be scored as recommend by the TIL-WG [2, 3] as a continuous variable, with clinically relevant cut-offs in mind.

Even though TILs will require validation in accordance with regulatory standards prior to be clinically recommended as a predictive biomarker for response to ICI, TILs≥5% have been shown to be predictive of response to pembrolizumab on the exploratory analysis of the randomized phase III KEYNOTE-119 clinical trial [57]. Additionally, TILs have been

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analytically validated, with three ring studies showing reliable inter-reader reproducibility [97-99], and have the advantage of being easily assessed on a simple H&E slide with an existing standardized method that is available to the pathology community though numerous publications and at the TIL-WG website [2, 89]. In a recent publication, an analysis of the most discordant cases on the ring studies identified possible pitfalls for scoring TILs, including technical factors, sample heterogeneity, variability in defining tumor boundaries, differentiating lymphocytes from mimics, and limited stroma for evaluation. Approaches to avoid these pitfalls have been covered in [97] and associated educational resources are available at the TIL-WG website [89]. Once pathologists score TILs in their daily practice for prognostic purposes, this information will already be present in the report. As shown by Liu *et al* using SP142 LDT, a significant proportion of PD-L1+ ICs are macrophages [48], while TILs are composed of lymphocytes and plasma cells. In addition to providing this biologically relevant predictive information, TILs can also serve as a starting point. It is improbable that a tumor with no TILs will be PD-L1+. Similarly, PD-L1 borderline cases are likely to have low TILs. At the same time, cases with high TILs are highly likely to be PD-L1+, as evidenced on the BEP of IMpassion130 exploratory analysis, in which virtually all cases with TILs>20% were PD-L1+ [24]. Therefore, used in combination with TILs it may conceptually not matter which PD-L1 assay is used, as long as it is validated according to international standards. TILs are highly likely to be the backbone of predictive and prognostic information.

In conclusion, pathologists have a responsibility to patients to implement assays that lead to the most optimal selection of patients for immunotherapies. Solving the current issues in implementation of PD-L1 assays in clinical trials and daily practice require a partnership between industry, academia and regulating agencies, involving patient advocates. Since TILs and

PD-L1 are part of an immunological spectrum in BC, and PD1-PD-L1 interaction is only one of many factors that may determine the clinical outcome of immunotherapeutic therapies, assessing both as a composite biomarker may be the best way to identify patients most likely to respond to ICI. However, reality and regulatory implementations dictate that practices will vary across different jurisdictions. We propose herewith a risk-management framework that may help mitigate the risks of suboptimal patient selection for immune-therapeutic approaches in BC.

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expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer / World Health Organization.

List of abbreviations

BC, breast cancer; CPS, combined positive score; BEP, biomarker evaluable population; EU, European Union; HR, hazard ratio; IC, immune cell; IC_A, PD-L1 positive immune cell area; ICC, interclass correlation coefficient; ICI, immune checkpoint inhibitor (used in this manuscript specifically for PD-1/PD-L1 inhibition-based therapy); IHC, immunohistochemistry; ITT, intention-to-treat; LDT, laboratory-developed test; NAC, neoadjuvant chemotherapy; NICE, National Institute for Health and Care Excellence; NCCN, National Comprehensive Cancer Network; NSCLC, non-small cell lung cancer; OPA, overall percent agreement; OS, overall survival; pCR, pathological complete response; PFS, progression-free survival; TC, tumor cell; TILs, tumor-infiltrating lymphocytes; TMB, tumor mutational burden; TNBC, triple negative breast cancer; TPS, tumor positive score

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Table 1: Technical details, scoring system and use on completed breast cancer clinical trials for each PD-L1 antibody.

	Commercial diagnostic assays used in clinical trials					Biosimilar diagnostic antibodies used in clinical practice
Assay	SP142	22C3	SP263	73-3	28-8	E1L3N (Cell Signalling), CAL10 (Zytomed), QR1 (Quartett), ZR3 (Cell Mark)
Binding epitope	C-terminus cytoplasmic domain	Discontinuous segments on the extracellular domain	C-terminus cytoplasmic domain	C-terminus cytoplasmic domain	Discontinuous segments on the extracellular domain	E1L3N: C-terminus cytoplasmic domain
Platform	Ventana BenchMark ULTRA	Agilent Link 48	Ventana BenchMark ULTRA	Agilent Link 48	Agilent Link 48	any
Scored cell type	IC	IC and TC	IC or TC	IC or TC	TC	depending on score
Scoring system	IC_A: $\dfrac{PD\text{-}L1^+IC}{tumor\ area}$	CPS: $\dfrac{PD\text{-}L1^+IC + PD\text{-}L1^+TC}{TC}$ TPS: $\dfrac{PD\text{-}L1^+TC}{TC}$	$T_\%$: $\dfrac{PD\text{-}L1^+TC}{TC}$ $IC_{IC\%}$: $\dfrac{PD\text{-}L1^+IC}{IC}$	$T_\%$: $\dfrac{PD\text{-}L1^+TC}{TC}$ $IC_{IC\%}$: $\dfrac{PD\text{-}L1^+IC}{IC}$	$T_\%$: $\dfrac{PD\text{-}L1^+TC}{TC}$	depending
Partner drug	Atezolizumab	Pembrolizumab	Durvalumab	Avelumab	Nivolumab	any
Breast cancer clinical trials	IMpassion130 NCT01375842 NCT01633970 KATE-2	KEYNOTE-119 KEYNOTE-150 KEYNOTE-086 KEYNOTE-012 PANACEA KEYNOTE-173 KEYNOTE-552 TONIC (Nivolumab)	GeparNuevo	JAVELIN		none

CPS: combined positive score; IC: immune cells; TC: tumor cells; TPS: tumor positive score.

Table 2: Prevalence of PD-L1 according to assay in breast cancer.

Study	Samples number and site	SP142	SP263	22C3	Others
Scott *et al* [22]	196 TNBC	$IC_A \geq 1\%$:32% $CPS \geq 1$: 35% $TC_\% \geq 1\%$: 11%	$IC_A \geq 1\%$:54% $CPS \geq 1$: 64% $TC_\% \geq 1\%$: 53%	$IC_A \geq 1\%$:51% $CPS \geq 1$: 60% $TC_\% \geq 1\%$: 50%	**28-8** $IC_A \geq 1\%$:46% $CPS \geq 1$: 52% $TC_\% \geq 1\%$: 35%
Noske *et al* [23]	30 primary TNBC samples	$IC_A \geq 1\%$: 50%	$IC_A \geq 1\%$: 87%	$IC_A \geq 1\%$: 57% $CPS \geq 1$: 60%;	**28-8** $IC_A \geq 1\%$: 63%
Noske *et al* [23]	104 primary TNBC samples	$IC_A \geq 1\%$: 44%	$IC_A \geq 1\%$: 82%		
Reisenbichler *et al* [25]	68-76 primary TNBC samples	$IC_A \geq 1\%$: 58% (n=68)	$IC_A \geq 1\%$: 78% (n=76)		
IMpassion130 NCT02425891 [24]	614 primary and metastatic TNBC samples	$IC_A \geq 1\%$: 46%	$IC \geq 1\%$:75%	$CPS \geq 1$: 81%	
IMpassion130 NCT02425891 [24, 27, 28]	902 primary and metastatic TNBC samples	All: $IC_A \geq 1\%$:41% primary: $IC_A \geq 1\%$:44% metastatic: $IC_A \geq 1\%$:36% All: $TC_\% \geq 1\%$: 9% (900)			
FDA SSED [14]	2744 primary and 50 metastatic TNBC samples	All: $IC_A \geq 1\%$:50% primary: $IC_A \geq 1\%$:50% metastatic: $IC_A \geq 1\%$:78%			
Carter *et al* [29]	500 chemotherapy naïve TNBC	$IC_A \geq 1\%$: 46% $TC_\% \geq 1\%$: 9%			
Downes *et al* [26]	30 BC	$IC_A \geq 1\%$:47-50%		$CPS \geq 1$: 53-63%%	**E1L3N:** $IC_A \geq 1\%$:53-63% $CPS \geq 1$: 53-67%%
NCT01633970 [30]	24 TNBC	$IC_A \geq 1\%$: 50% $TC \geq 1\%$:17% (of which 92% were $IC_A \geq 1\%$)			
NCT01375842 [7]	112 TNBC	$IC_A \geq 1\%$: 78%*			
KEYNOTE-119 NCT02555657 [31]	622 TNBC			$CPS \geq 1$: 65% $CPS \geq 10$: 31% $CPS \geq 20$: 18%	
KEYNOTE-012 NCT01848834 [32]	111 TNBC			$CPS \geq 1$: 59%	
KEYNOTE-086	170 primary and metastatic			$CPS \geq 1$: 62%	

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NCT02447003 [33]	samples TNBC				
KEYNOTE-150 NCT02513472 [34]	107 TNBC			CPS≥1: 46%	
JAVELIN NCT01772004 [35]	136 BC, 48 TNBC				**73-3** All: IC≥10%: 9% TNBC: IC≥10%: 19%
TONIC NCT02499367 [36]	70 metastatic TNBC samples			IC≥1%: 86% IC≥5%: 67%	
GeparNuevo NCT02685059 [37]	158 TNBC		$IC_{IC\%}$ and/or TC%≥1%: 87%		

CPS: combined positive score; FDA SSED: Food and Drug Administration summary of safety and effectiveness data; IC: immune cells; IC_A: PD-L1-positive immune cell area; met: metastatic or non-primary sample; n: number of patients included in the analysis; prim: primary sample; TC: tumour cells; TNBC: triple negative breast cancer.

* The first 25 patients were selected only if PD-L1+, then enrolment was extended to all patients, explaining the higher PD-L1 prevalence.

Table 3: Studies evaluating inter-reader reproducibility on breast cancer samples.

Study	Assay and scoring	participating pathologists	Samples evaluated	Training	Concordance
Reisenbichler *et al* [25]	SP142 CDA $IC_A \geq 1\%$	19	68 primary TNBC	No specific training for the study.	ICC 0.560, OPA 41%
	SP263 CDA $IC_A \geq 1\%$				ICC 0.513
Noske *et al* [23]	SP142 CDA $IC_A \geq 1\%$	7	30 primary TNBC	Trained on digital platform for the evaluation of PD-L1 IC with SP142 and had to pass a proficiency exam.	ICC 0.805
	SP263 CDA $IC_A \geq 1\%$				ICC 0.616
	22C3 CDA $IC_A \geq 1\%$				ICC 0.605
	28-8 CDA $IC_A \geq 1\%$				ICC 0.460
FDA SSED [14]	SP142 CDA $IC_A \geq 1\%$	3	60 TNBC	Not specified.	OPA 91.1%
Dennis *et al* [49]	SP142 CDA $IC_A \geq 1\%$	903	28 TNBC	Regional trainer lead sessions and digital platform training conducted by Roche International Pathologist Training program. A proficiency test was evaluated.	OPA 98%
Downes *et al* [26]	SP142 CDA $IC_A \geq 1\%$	3	30 BC	Not specified.	ICC 0.956, OPA 98%
	22C3 CDA $CPS \geq 1$				ICC 0.862, OPA 93%
	E1L3N LDT $IC \geq 1\%$				ICC 0.862, OPA 93%
	E1L3N LDT $CPS \geq 1$				ICC 0.815, OPA 91%
Solinas *et al* [50]	E1L3N LDT $IC \geq 1\%$	2	441 BC	Not specified.	ICC 0.10-0.58 for primary treatment naïve tumours, ICC 0.94[0.84-0.97] for NAC treated, ICC 0.00 [-0.54-0.35] for relapses

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CDA: commercial diagnostic assay; FDA SSED: Food and Drug Administration summary of safety and effectiveness data; ICC: Interclass correlation coefficient; LDT: laboratory developed test; OPA: overall percent agreement.

Note: Definition of methodology for OPA, i.e. whether all individual pathologists were compared or if the mean test score for all pathologists was compared to the consensus, was not available for all the studies in Table 3 precluding fair comparison among studies.

Table 4: Studies evaluating clinical validity and utility of PD-L1 IHC and/or TILs as a predictive biomarker of response to PD1/PD-L1 inhibitors in breast cancer.

Clinical trial	Drug	Tumor type (n)	Biomarker details (n)	Predictive capacity of PD-L1/TILs
Advanced setting				
IMpassion130 NCT02425891 [24, 27, 28]	nab paclitaxel +/- atezolizumab randomized phase III	UnTx LAdv or mTNBC (902)	PD-L1 (SP142) was prospectively tested at BTx (902) and used as a stratification factor for randomization. TILs were evaluated retrospectively (460 and 614). PD-L1 SP263 and 22C3 were performed retrospectively on BEP (614) post-hoc exploratory analysis.	Improved PFS (HR 0.62 [0.49-0.78]) and OS (HR 0.62 [0.45-0.86]) with the addition of atezolizumab in PD-L1+ tumors (SP142 $IC_A \geq 1\%$). ORR 56 vs 46% in the ITT population and 59 vs 43% in PD-L1+ tumors (p=0.002). Better PFS (0.53 [0.38-0.74]) and OS (0.57 [0.35-0.92]) for TIL>10%PD-L1≥1% population (n=460). PD-L1+ cases showed higher median TILs (10% [IQR:5-20]) on BEP. Improved PFS and OS (0.64 [0.53-0.79]; 0.75 [0.59-0.96]) with the addition of atezolizumab in SP263 (IC≥1%) and (0.68 [0.56-0.82]; 0.78 [0.62-0.99]) 22C3 (CPS≥1) on BEP. Median PFS SP142 4.2 months, 22C3 2.1 months, SP263 2.2 months, and median OS SP142 9.4 months, 22C3 2.4 months, SP263 3.3 months.
NCT01375842 [7]	atezolizumab single arm phase Ib	PreTx mTNBC (116)	PD-L1 (SP142) tested prospectively (116). TICs (116).	PD-L1 $IC_A \geq 1\%$ (ORR:12 vs 0%; HR: 0.55 [0.33-0.92]) and TICs>10% (HR:0.54 [0.35-0.83]) were associated with better outcome. TICs>10% was independently associated with ORR, PFS and OS in multivariate analysis. PD-L1 TC≥1% was not associated with response.
NCT01633970 [30]	atezolizumab + nab paclitaxel single arm phase Ib	UnTx (13) and PreTx (20) mTNBC (33)	PD-L1 (SP142) and TILs tested retrospectively at BTx (23 and 20) and PostTx (11 and 15, respectively).	No statistically significant association of baseline PD-L1 or TILs with response. Numerically higher ORR (41.7 vs 33.3%) and longer PFS (6.9 vs 5.1 mo) and OS (21.9 vs 11.4 mo) in PD-L1+ tumor ($IC_A \geq 1\%$). Numerically higher OS in TILs>5%. Changes in PD-L1 or TILs were not associated with clinical response.
KEYNOTE-119 NCT02555657 [31, 55]	physician's choice chemo +/- pembrolizumab randomized phase III	PreTx mTNBC (622)	PD-L1 (22C3) was prospectively tested at BTx (622) and used as a stratification factor for randomization.	No improved outcome in ITT population or PD-L1+ tumors (CPS≥10 p=0.057; CPS≥1 p=0.073). For CPS≥20, HR OS: 0.58 [0.38-0.88]. Better OS for TILs≥5% (0.75 [0.59-0.96]) in the pembrolizumab arm but not the chemotherapy arm (1.46 [1.11-1.92]). TILs and PD-L1 CPS moderately correlated (0.45). TILs (p=0.004) and CPS (p=0.09) were independently predictive.
KEYNOTE-150 NCT02513472	pembrolizumab + eribulin single arm	PreTx and UnTx mTNBC	PD-L1 (22C3)	ORR independent (30.6 vs 22.4%) of PD-L1 status (CPS≥1).5

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[34]	phase Ib/II	(107)		
KEYNOTE-086 NCT02447003 [33, 56, 57]	pembrolizumab single arm phase II	A: PreTx mTNBC (170) B: UnTx PD-L1+ mTNBC (84)	PD-L1 (22C3) was prospectively tested at BTx (254). TILs were evaluated retrospectively (193).	ORR independent of PD-L1 status (CPS≥1) on cohort A (5.7 vs 4.7%). No difference in PFS or OS between PD-L1+ and PD-L1-. 21.4% ORR cohort B. Better ORR in pts with TILs>median in cohort A (6 vs 2%) and B (39 vs 9%) and combined cohorts (OR: 1.26 [1.03-1.55]). Higher median Higher TILs in responders vs non-responders in cohort A (10% vs 5%) and cohort B (50% vs 15%).
KEYNOTE-012 NCT01848834 [32]	pembrolizumab single arm phase Ib	PreTx PD-L1+ mTNBC (32)	PD-L1 (22C3) was prospectively tested at BTx (32)	Increasing ORR (p=0.028) and reduction in HR (p=0.012) with increasing PD-L1 expression.
TONIC NCT02499367 [36]	nivolumab +previous induction therapy (Rx/chemo) randomized phase II	PreTx and UnTx mTNBC (67)	PD-L1 (22C3) and TILS at BTx, after induction and PostTx.	Higher BTx TILs (median 12.5 vs 6%, p=0.004) and PD-L1 on IC (median 15 vs 5%) on responders vs non-responders. Better PFS and OS was observed in PD-L1 IC≥5% patients. No difference was observed between PD-L1 TC≥1% and <1% populations.
PANACEA NCT02129556 [58]	pembrolizumab + trastuzumab single arm phase Ib/II	PreTx LAdv or mHER2+ BC Ib: PD-L1+ (6) II: PD-L1+ & PD-L1- (52)	PD-L1 (QualTek/ 22C3) tested prospectively at BTx (58). TILs were evaluated retrospectively (48).	II: Higher ORR (15 vs 0%) in PD-L1+ (CPS≥1). Longer OS for PD-L1+ population. Higher TILs levels in objective responders (median ~25 vs 1.5% p=0.006) and in PD-L1+ population (p=0.0004).
KATE-2 NCT02924883 [59]	T-DM1+/- atezolizumab randomized phase II	PreTx LAdv or mHER2+ BC (202)	PD-L1 (SP142) tested prospectively (202).	PFS survival benefit (HR 0.60[0.32-1.11]) and numerically higher ORR (54 vs 33%) in PD-L1+ tumors (IC$_A$≥1%) with the addition of atezolizumab.
JAVELIN NCT01772004 [35]	avelumab single arm phase Ib	PreTx LAdv or mBC (168)	PD-L1 (73-10) evaluated prospectively on IC and TC (168).	Better ORR in PD-L1+ (IC≥10%) BC (16.7 vs 1.6% p=0.039, 22.2 vs 2.6% in TNBC). No association between outcome and PD-L1+ (HR PFS: 0.66[0.34-1.26], OS: 0.62 [0.25-1.54]). PD-L1 on TC showed no association with response.
Neoadjuvant setting				
KEYNOTE-552 NCT03036488 [60]	Neoadjuvant paclitaxel + carboplatin + AC/EC +/- pembrolizumab randomized phase III	UnTx TNBC (602)	22C3	pCR achieved irrespective PD-L1 status (CPS≥1) with the addition of pembrolizumab (68.9 vs 45.3%).
KEYNOTE-173 NCT02622074	Neoadjuvant pembrolizumab + nab-paclitaxel	UnTx LAdv TNBC	PD-L1 (22C3) retrospectively tested on BTx	Higher BTx (p=0.028) and OnTx TILs (p=0.005) and BTxPD-L1 CPS (p=0.021) were associated with pCR. Responders had

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[61, 62]	+/- carboplatin +/-AC randomized phase Ib	(60)	(52). TILs were retrospectively evaluated on BTx (53) and OnTx (50) samples.	higher median pre (40% vs 10%) and OnTx (65% vs 22.5%) TILs.

AC: doxorubicin + cyclophosphamide; BEP: biomarker evaluable population; BTx: baseline or pre-treatment; CPS: combined positive score; Δ: change between baseline and after treatment; EC: epirubicin + cyclophosphamide; HR: hazard ratio; IC: immune cells; IQR: interquartile range; LAdv: unresectable locally advanced; ITT: intention-to-treat population; mBC: metastatic breast cancer, all subtypes; mTNBC: metastatic TNBC; n: number of patients included in the analysis; OnTx: on treatment; OR: odds ratio; ORR: objective response rates; OS: overall survival; PostTX: post-treatment; PreTx: previously treated; PFS: progression free survival; Rx: radiation; TIC: tumor infiltrating immune cells (lymphocytes, macrophages, dendritic cells and granulocytes) scored as a percentage of tumor area; TC: tumor cells; UnTx: untreated.

Table 5: Risks associated with the integration of PD-L1 as immuno-oncological biomarkers for clinical trials and the daily practice.

Risk	Description of risk	Mitigation approach/Recommendation
Risks to patient safety		
Provision of inappropriate treatment because of false-positive or false-negative test results	Inter-pathologist variability and use of different assays with different sensibilities may mislead categorization of PD-L1 status. Incorrect results lead to inappropriate treatment allocation and put patient safety at risk.	See below.
Physical harm or inconvenience associated with tissue biopsy	Heterogeneity of PD-L1 expression between primary and metastatic lesions in TNBC [14] can lead to misleading categorization depending on the sample tested.	Define optimal sample for PD-L1 testing from data of future clinical trials. When both primary and metastatic samples available, test both if possible.
Operational risks		
Failure of sample collection, processing and quality	Poor quality samples can result in unreliable test results.	Ensure correct sample fixation for 6 to 72hs and processing. Determine sample adequacy on H&E: presence of TC and tumor-associated IC. Cut 4um sections for PD-L1 IHC testing along with sections for other IHC to preserve tissue in biopsy samples. Use within 2 months of cutting [14].
Within laboratory assay variability	Drifts in assay results over time can result in unreliable test results.	Follow staining protocol with optimized conditions. Include control tissue (tonsil) to test acceptance criteria [14]. Internal and external quality assurance. Audit positivity rates [85].
Risks to biomarker development		
Difference in PD-L1 expression prevalence among assays	SP142 has shown PD-L1 expression on a lower number of TC and IC compared to the other assays [9, 10, 12, 13, 16, 22-24, 38-42].	It is more important that an assay identifies the patients who will most likely respond, than identifying a greater proportion of PD-L1 positive patients. Even though assays are not analytically equivalent, clinical utility interchangeability must be further studied.
Use of multiple scoring systems	The existence of multiple scoring systems for the PD-L1 assays preclude the homologation of assays and complicate reproducibility.	For BC, PD-L1 expressed in IC and not in TC has been shown to be predictive of response [7, 27, 35, 36]. Future clinical trials should evaluate the most effective PD-L1 scoring system. Cut-points must be reproducible.
Inter pathologist variability to read assay	Quantification of PD-L1 on IC has been shown not be reproducible to expected standards [10-13, 18, 23, 38, 39].	Ensure training on expected staining profile and cut-off for pathologist participating in clinical trials. Use of a single scoring system. Automated quantification by computer-based image analysis. Evaluate interobserver variability with a sufficiently large and statistically powered number of

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		pathologists to ensure reproducibility.
Temporal and Spatial heterogeneity	Both PD-L1 and TILs have demonstrated higher expression in primary tumors than in metastases [2, 24, 57].	Evaluating all available samples on clinical trials would provide useful data, since the most appropriate time point for testing has not yet been clearly established.
Unique biomarker as companion diagnostic test	Due to the complexity of immune response it is unlikely a single biomarker will sufficiently predict response to ICI.	Since both PD-L1 and TILs have shown to be predictive of response to ICI [28,57], the use of both as stratification factors and for composite biomarker analysis in future clinical trials may help further optimize patient selection. Enough samples should be secured to further investigate other biomarkers on exploratory analysis.
Risks to biomarker implementation into daily practice		
Regulatory approval differs per country	Implementation into daily practice is dependent on regulatory approval.	Thorough and timely scientific interaction between the pathology community, industry and regulatory and national reimbursement agencies is needed.
Biomarker accessibility and affordability	PD-L1 testing is not yet covered by health insurance in many countries.	Thorough and timely scientific interaction between the pathology community, industry and regulatory and national reimbursement agencies is needed.
Use of multiple PD-L1 assays for a single analyte	With multiple PD-L1 assays available, pathology labs cannot be expected to have all tests available, causing variability in test results between laboratories.	Choice of assay will depend on regional regulations, availability of antibody, automated staining platform and optimized assay in currently in use. Consider LDTs. Outsource to reference laboratories.
Difference in PD-L1 expression prevalence among assays	SP142 has shown PD-L1 expression on a lower number of TC and IC compared to the other assays [9, 10, 12, 13, 16, 22-24, 38-42].	It is more important that an assay identifies the patients who will most likely respond, than identifying a greater proportion of PD-L1+ patients. For BC SP142, SP263 and 22C3 have shown to identify patients that derive better outcome in response to atezolizumab and nab-paclitaxel [24].
Inter pathologist variability to read assay	Quantification of PD-L1 on IC has been shown not be reproducible to expected standards [10-13, 18, 23, 38, 39].	Training on expected staining profile and cut-off. Interpretation guideline. Use of a single scoring system. Automated quantification by computer-based image analysis.
Unique biomarker as companion diagnostic test	Due to the complexity of immune response it is unlikely a single biomarker will sufficiently predict response to ICI. The use of PD-L1+ IC score as a unique biomarker test maybe suboptimal in real world conditions.	Since TILs and PD-L1 are part of an immunological spectrum and PD-1/PD-L1 interaction is only one of many factors that may determine the clinical outcome of immunotherapeutic therapies, assessing both as a composite biomarker could be a better way to identify patients most likely to respond to ICI.

H&E: hematoxylin and eosin; IC: immune cells; IHC: immunohistochemistry; LDTs: laboratory developed test; PD-L1+ IC: proportion of tumor area covered by IC with discernible PD-L1 staining of any intensity expressed as a

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percentage; ICI: PD-1/PD-L1 inhibition based therapy; TC: tumor cell; TILs: tumor infiltrating lymphocytes; TNBC: triple negative breast cancer.