PD-L1 evaluation in the gastrointestinal tract: from biological rationale to its clinical application

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Summary

Immune-checkpoint inhibitors targeting the PD-1/PD-L1 axis have brought significant clinical benefit in many solid cancer types, including gastrointestinal malignancies. However, it has been estimated that only 20-40% of patients respond to treatment. The pattern of expression and potential predictive value of PD-L1 as an immunohistochemical biomarker has been extensively studied in gastrointestinal neoplasms. Until now, its predictive value has been demonstrated, and is currently in use only in upper gastrointestinal malignancies (gastroesophageal adenocarcinoma and esophageal squamous cell carcinoma).
In this Review, we describe the technical aspects and challenges related to PD-L1 immunohistochemical assays, the current role of PD-L1 as a biomarker in clinical practice and we outline the main studies and clinical trials analyzing the prognostic and predictive value of PD-L1 in gastrointestinal cancers.

Key words: PD-L1, gastrointestinal neoplasms, clinical trials, immunotherapy, immunohistochemistry

Introduction

Rudolf Virchow, the father of modern pathology, described the "lymphoreticular infiltrate" in neoplastic tissues in 1863, and hypothesized that there was a connection between cancer and inflammation [1]. On this basis, more than a century of research has shed light on the complex interaction between cancer and the host immune system, as the latter exerts antitumor activity by activation of the innate and adaptive response. However, during the process of tumor immune editing, cancer cells develop several methods of escaping the host immune response, establishing an immunosuppressive tumor microenvironment (TME) [2]. Landmark studies have demonstrated that programmed death 1 (PD-L1)/programmed ligand death 1 (PD-L1)-mediated immune checkpoint has a crucial role in tumor immune escape [3]. PD-1 was first described in

1992 by a group from Kyoto University as an apoptosis-associated gene [4]. Further studies from the same group identified that PD-1 expression was found on the surface of T and B lymphocytes and was involved in the inhibition of immune response [5,6].

PD-1 is a checkpoint protein and suppressor T-cell receptor and is part of the CD28 family. It is expressed by T and B cells, monocytes and dendritic cells. PD-L1 is a transmembrane glycoprotein of the B7 ligand family commonly expressed on the surface of antigen-presenting cells and cancer cells. Multiple signaling pathways have been identified as regulators of PD-L1 expression on tumor cells, including NFκB, MAPK, mTOR, STAT and c-Myc [7].

The binding of PD-L1 to PD-1 on T cells causes dephosphorylation of the T-cell receptor SHP-1/2, which in turn results in phosphorylation of the downstream proteins spleen tyrosine kinase (Syk) and phospholipid inositol-3-kinase (PI3K), inhibiting downstream signaling. PD-1/PD-L1 axis activation: i) reduces T cell-mediated immune surveillance, ii) diminishes tumor-infiltration of CD4+/CD8+ T cells, and iii) reduces cytokines including tumor necrosis factor (TNF), interferon-γ (IFN-γ) and Interleukin-2 (IL-2). Overall, this interaction leads to T cell exhaustion and apoptosis, allowing tumor cells to escape immune surveillance [7].

In 2010 a landmark clinical study showed striking effects of immunotherapy for the first time: in a compassionate-use trial in patients with advanced refractory melanoma, the use of Ipilimumab, a monoclonal antibody that antagonizes CTLA-4, resulted in significant clinical benefit [8].

Monoclonal antibody inhibitors of the PD-1/PD-L1 axis (*i.e.*, immune checkpoint inhibitors [ICIs]) have induced remarkable clinical benefits at advanced stages in various cancer types, becoming the backbone of cancer immunotherapeutic strategies. There are currently 5,683 ongoing/completed clinical trials testing anti-PD1/PD-L1monclonal antibodies, alone or in combination with other therapeutic agents [9].

Until now, the Food and Drug Administration (FDA) has approved six monoclonal antibodies targeting PD-1 (Nivolumab, Pembrolizumab and Cemiplimab) or PD-L1 (Atezolizumab, Durvalumab and Avelumab) for the treatment of various cancer types, including melanoma, non-small cell lung cancer, breast cancer, gastroesophageal and colorectal cancer [10]. However, only a subset of patients benefits from PD-1/PD-L1 blockade, with response rates lower than 40%. Most patients show primary resistance to single-agent ICI therapy, and longer follow-up of clinical trial populations is now revealing the development of acquired resistance [11]. Thus, future efforts should focus on elucidating resistance mechanisms, exploring effective predictive biomarkers, and developing novel combinational therapies.

PD-L1 testing in gastrointestinal cancers

At present, microsatellite instability (MSI)/mismatch repair protein deficiency (MMRd) and PD-L1 expression are the only predictive biomarkers approved for the use of immunotherapy in the context of gastrointestinal malignancies [12].

Pembrolizumab is currently in use for the treatment of patients with unresectable or metastatic, MSI/MMRd solid tumors that have progressed following prior treatment and who have no satisfactory alternative treatment options. In colorectal and gastroesophageal adenocarcinomas, ICIs are used as first-line treatment in patients with metastatic or unresectable MSI/MMRd tumors [13,14].

The pattern of expression and potential predictive value of PD-L1 has been extensively studied in gastrointestinal neoplasms. However, its role as a predictive biomarker has been demonstrated and is currently in use only for upper gastrointestinal tumors, namely: esophageal squamous cell carcinoma, esophageal and gastroesophageal junction adenocarcinoma and gastric adenocarcinoma [14]. For these cancer types, the expression of PD-L1 should be evaluated using the Combined Positive Score (CPS), which consists in dividing the number of positive tumor cells, lymphocytes and macrophages, by the total number of viable tumor cells multiplied by 100 [15]. Of note, tumor cells with cytoplasmic staining, neutrophils, eosinophils, plasma cells, stromal cells, necrotic cells and cellular debris should be excluded from the numerator when calculating the CPS. The alternative method of calculating PD-L1 score, currently in use for non-small cell lung cancer, is the Tumor Proportion Score (TPS), which is defined as the number of positive tumor cells divided by the total number of viable tumor cells multiplied by 100% (Fig. 1). In gastroesophageal adenocarcinomas, the CPS has been shown to outperform TPS in predicting response to ICIs [15,16]; however, a recent study by Doki et al. [17] demonstrated that both TPS and CPS can be useful predictive biomarkers in esophageal squamous cell carcinoma and should thus be included in the pathology report. The cut-off value of CPS to identify patients who might benefit from immunotherapy is subject to change. While the previous cut-off value for the use of ICIs in gastroesophageal adenocarcinomas was set to CPS ≥ 1, recent subgroup analyses of clinical trials support the use of Pembrolizumab

for CPS ≥ 10 and Nivolumab for CPS ≥ 5. For this reason, the pathologist should indicate in the pathology report the exact CPS score when assessing PD-L1 expression in gastroesophageal adenocarcinoma specimens or should specify a clinically meaningful interval (i.e. CPS < 1; 1-4; 5-9; ≥ 10) in order to allow the oncologist to choose the best therapeutic option for the patient (Fig. 2) [14]. For esophageal carcinoma,

the TPS evaluation should be also reported, according to recent data.

PD-L1 expression can be assessed by using different immunohistochemical (IHC) assays, each consisting of a specific anti-PD-L1 antibody clone, a platform, and a scoring system. The pathology report should indicate all this information, to ensure that PD-L1 evaluation in clinical practice is comparable to the assess-
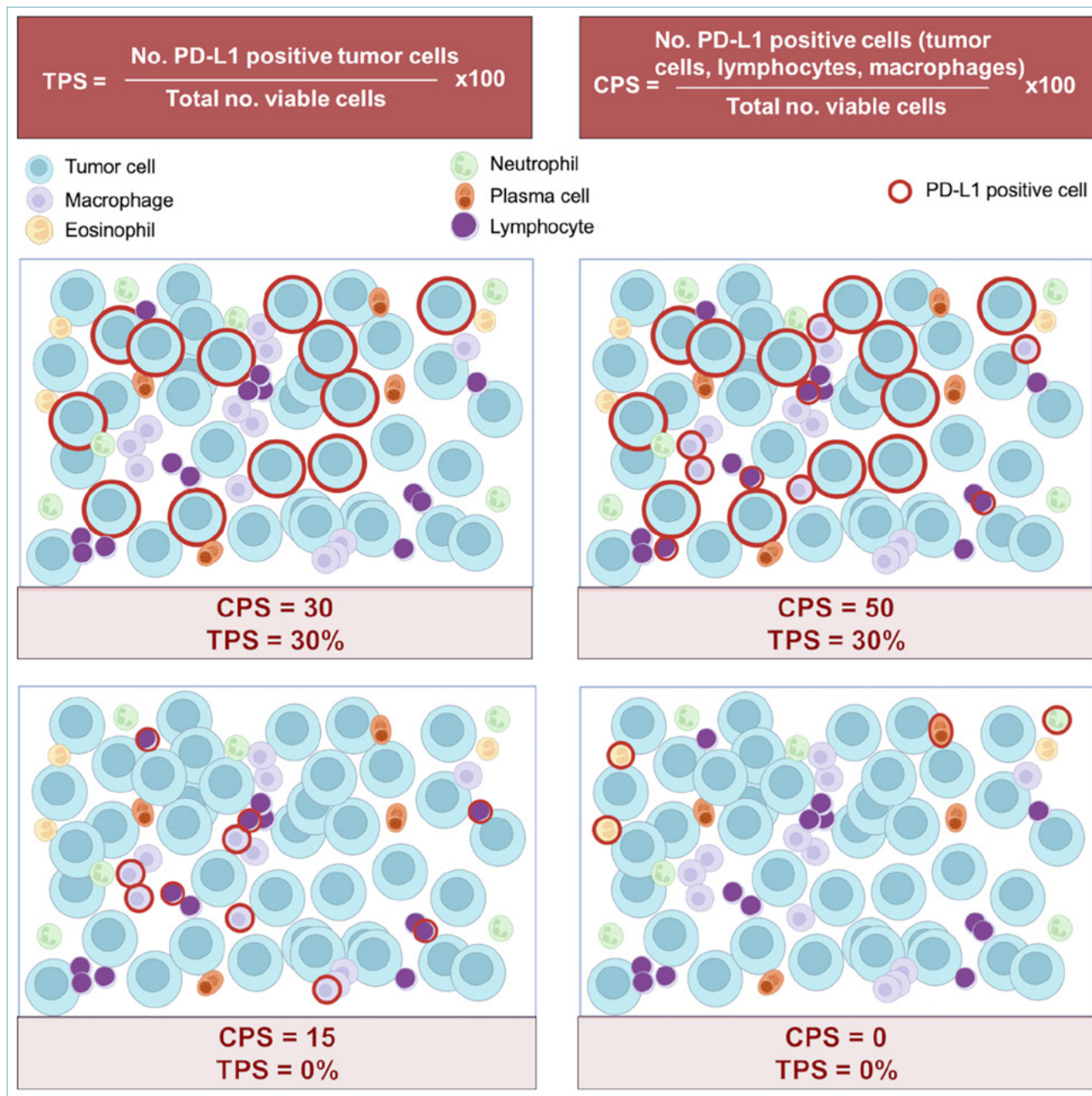

Figure 1. Examples of PD-L1 immunohistochemistry scoring using Combined Positive Score (CPS) and Tumor Proportion Score (TPS).

ment within the related clinical trials [18]. Of note, a distinctive requirement of the different PD-L1 IHC assays is the use of different autostainers. FDA-approved PD-L1 assays are classified as companion diagnostics. The FDA defines an assay as a companion diagnostic if it provides information that is "essential for the safe and effective use of a corresponding drug or biological product". The FDA has approved the use of three PD-L1 IHC assays as companion diagnostics: *Dako 22C3* for Pembrolizumab in patients with several solid tumors, including gastroesophageal adenocarcinoma; *Ventana SP142* for Atezolizumab in patients with uro-
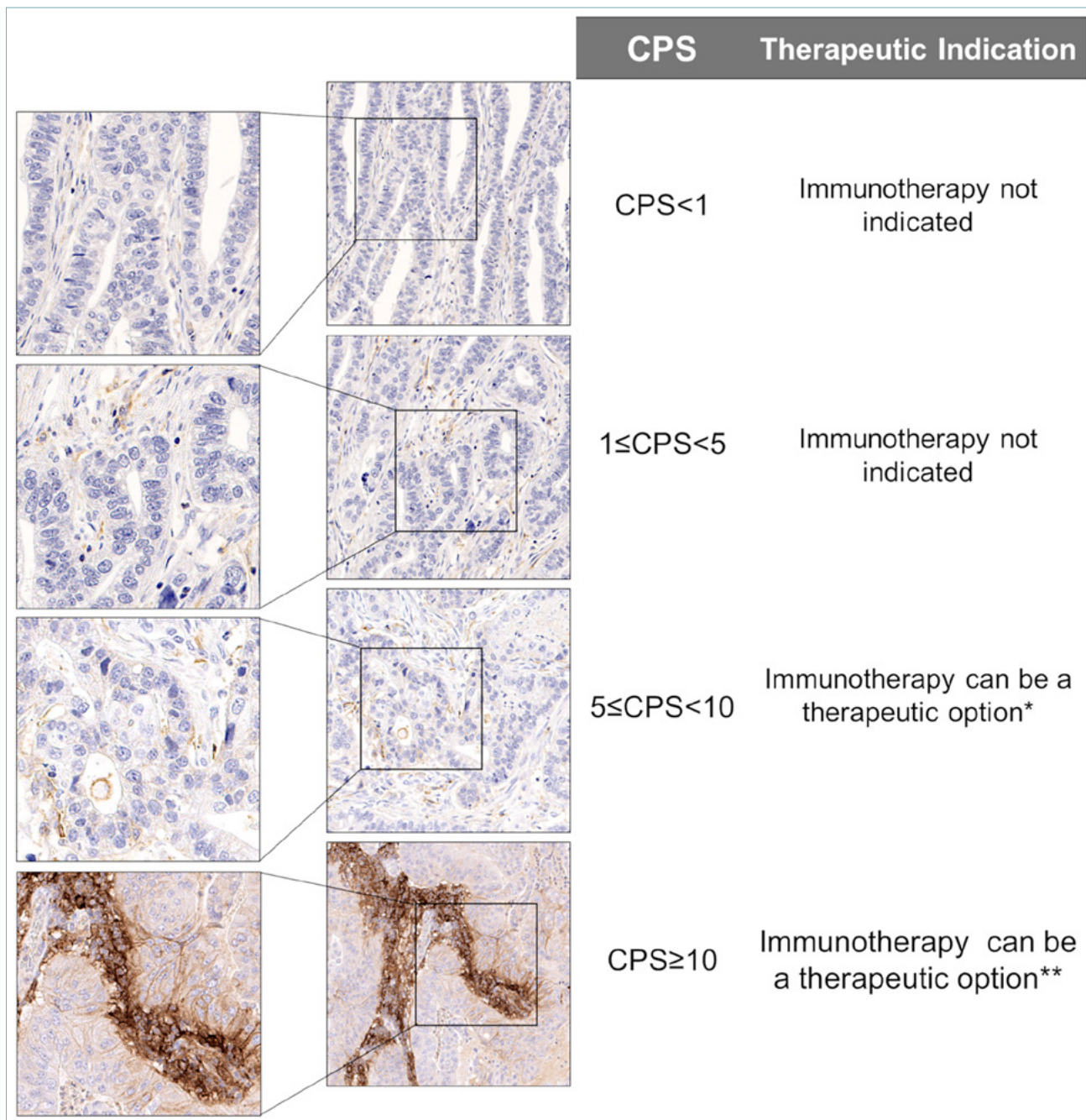

Figure 2. PD-L1 expression staining patterns by Combined Positive Score (CPS) and relative therapeutic indications in gastroesophageal adenocarcinoma. *At present, Nivolumab is indicated (Checkmate 649). **At present, Nivolumab (Checkmate 649) or Pembrolizumab (Keynote 590) are indicated.

thelial carcinoma, triple-negative breast cancer or non-small-cell lung cancer; and *Dako 28-8* for the combination of Ipilimumab and Nivolumab in patients with NSCLC [19]. Although a study provided evidence for the potential interchangeability of 22C3 PD-L1 clone (used in the KEYNOTE-061 study) and 28-8 PD-L1 clone (used in the CheckMate 649 study) [20], another recent study showed only moderate concordance rate between the two assays, with higher sensitivity of the 28-8 PD-L1 clone [21]. Moreover, several studies provided evidence that different PD-L1 assays (SP142, E1L3N, 28-8, SP263, 22C3) have only low to moderate concordance rates [22-24]. Therefore, the predictive value of different PD-L1 clones is still controversial.

PD-L1 evaluation can be challenging under certain circumstances and the pathologist must be aware of potential pitfalls. First of all, a correct evaluation of PD-L1 expression can be hampered by intra-tumoral heterogeneity. A study that used tissue microarray to analyze multiple cores from the same gastric cancer specimen found inconsistency in PD-L1 expression [25]. The results of the same study indicated that at least five biopsies are required to achieve a good representation of the tumor in terms of PD-L1 expression [25]. Additionally, when evaluating PD-L1 expression, areas of ulceration or chronic inflammatory processes (*i.e.,* chronic gastritis) should be excluded from the CPS evaluation. A recent study has investigated PD-L1 expression patterns in gastroesophageal dysplastic lesions and found a relatively high prevalence of PD-L1 positivity among these lesions, stressing the importance to make a proper distinction between pre-invasive lesion and invasive carcinoma [26]. A robust interobserver agreement is necessary to guarantee the reproducibility of PD-L1 assay. The manufacturers of the 22C3 assay reported an overall percentage agreement of 96.6% when assessing gastroesophageal cancer samples [15]. However, results from an independent group showed less-robust reproducibility, with intraclass correlation coefficients (ICCs) of 0.39 and 0.26 for a CPS of ≥ 1 and of 0.23 and 0.14 for a CPS of ≥ 10, using the 22C3 and SP263 clones, respectively [27]. These data stress the need for additional training for practicing pathologists and for additional studies on the reproducibility of the PD-L1 assays and scoring system. Another issue to consider is the lack of consistency of PD-L1 immunohistochemistry using formalin-fixed paraffin-embedded tissue older than five years.

PD-L1 in gastroesophageal cancers

Recent clinical trials have shown that esophageal, esophagogastric junction, and gastric cancer benefit from treatment with ICIs [14]. These emerging data have led to the recent approval of ICIs for the treatment of oesophageal, gastroesophageal and gastric cancer by the FDA and the European Medicines Agency (EMA). Accordingly, the European Society for Medical Oncology (ESMO) has recently integrated checkpoint inhibitors targeting PD-1, namely Nivolumab and Pembrolizumab, into the current clinical guidelines, as standard-of-care for esophageal, gastroesophageal and gastric cancer [28,29]. A summary of the current recommendations by the ESMO Clinical Practice Guidelines is presented in Table I. Besides these recommendations, the FDA has recently approved the use of Pembrolizumab for advanced unresectable or metastatic HER2 positive esophagogastric/gastric cancer patients in the first line setting, in combination with chemotherapy and Trastuzumab therapy [30].

In most cases, PD-L1 expression should be evaluated using the CPS scoring method. An exception is represented by the evaluation of PD-L1 in advanced/metastatic unresectable esophageal cancer, where the use of the TPS is recommended. PD-L1 positivity is defined as CPS ≥ 1 and TPS ≥ 1 [15]. For predictive purposes, the threshold to select patients for immunotherapies is different according to tumor location, type of drug to be used and line of treatment: for advanced/metastatic unresectable esophageal cancer in first-line treatment the threshold to select patients for immunotherapy is TPS ≥ 1% for Nivolumab therapy, but CPS ≥ 10 if Pembrolizumab therapy is considered. For advanced/metastatic unresectable upper GI adenocarcinoma in first-line treatment, the cut-off used is CPS ≥ 5 (Tab. I) [28,29].

PD-L1 expression occurs in approximately 50-60% of gastroesophageal or gastric adenocarcinoma cases [15,31] and 20-80% of esophageal squamous cell carcinomas [32-34]. PD-L1 upregulation is more frequent in immune cells of the tumor microenvironment, especially at the invasive margin, rather than in tumor cells in gastric and esophagogastric adenocarcinoma. If only tumor cells [35,36] are considered, the percentage of positive gastroesophageal and gastric cancer cases drops to 10-20% [24,37]. However, in esophageal squamous cell carcinoma cases, PD-L1 expression is more frequent in tumor cells rather than in immune or stromal cells [34]. The value of PD-L1 expression as a prognostic biomarker is still controversial [38].

Gastric cancer is a heterogenous tumor from the morphological and molecular standpoint and tumor heterogeneity should be taken into account for the evaluation of predictive biomarkers. Indeed, PD-L1 expression may vary across distinct morphological and molecular subgroups.

Table I. Current recommendations for immune checkpoint inhibitors therapy in esophageal, esophagogastric junction and gastric cancer according to the ESMO Clinical Practice Guidelines [28,29].

PD-1 inhibitor	Esophageal SCC	Esophageal ADC and EGJ carcinoma	Gastric cancer	Reference clinical trials
Nivolumab	**Adjuvant treatment** Monotherapy *Indications* Resectable, locally advanced disease Residual pathological disease after CRT and surgery	**Adjuvant treatment** Monotherapy Indications Resectable, locally advanced disease Residual pathological disease after CRT and surgery	/	CheckMate-577
	1st line treatment Combination with ChT/ Ipilimumab *Indications* Advanced/metastatic unresectable disease TPS ≥ 1%	**1st line treatment** Combination with ChT *Indications* Advanced/metastatic unresectable disease HER2 negative CPS ≥ 5	**1st line treatment** Combination with ChT *Indications* Advanced/metastatic unresectable disease HER2 negative CPS ≥ 5	CheckMate-648 CheckMate-649
	2nd line treatment Monotherapy	/	/	ATTRACTION-3
Pembrolizumab	**1st line treatment** Combination with ChT *Indications* Advanced/metastatic unresectable disease HER2 negative CPS ≥ 10	**1st line treatment** Combination with ChT *Indications* Advanced/metastatic unresectable disease HER2 negative CPS ≥ 10	/	KEYNOTE-590
	2nd line treatment Monotherapy *Indications* CPS ≥ 10	**/2nd line treatment** Monotherapy *Indications* CPS ≥ 10	**2nd line treatment** Monotherapy *Indications* MSI/dMMR gastric cancer	KEYNOTE-181 KEYNOTE-158

From a morphological point of view, PD-L1 is more frequently expressed in intestinal and mixed type gastric adenocarcinomas (54% and 56%, respectively), as compared to diffuse gastric cancer (32%) [39]. Within the rare histopathological variants recognized by the WHO classification, gastric carcinoma with lymphoid stroma (also known as medullary carcinoma or lymphoepithelioma-like carcinoma) is of particular interest when considering morphological biomarkers for targeted immunotherapies: the majority of gastric carcinomas with lymphoid stroma show expression of PD-L1 in tumor (33-68%) and/or immune (77-92%) cells [40,41].

Within the molecular subtypes proposed by The Cancer Genome Atlas (TCGA) research network [42], microsatellite instability (MSI) and Epstein-Barr virus (EBV) infection represent potential molecular hallmarks of response to immunotherapy and are the molecular subgroups more frequently associated with PD-L1 expression [39,43]. PD-L1 expression occurs in tumor and immune cells in up to 33% and 46%, respectively, of MSI gastric cancers [44,45]. Regarding EBV-associated gastric carcinomas, genomic amplification of the chromosomal region 9p24.1, the locus of genes encoding

PD-L1 and PD-L2, has been identified in about 15% of cases [42,45]. Although the highest levels of PD-L1 mRNA and protein expression have been identified in EBV-associated cases with 9p24.1 amplification, PD-L1 expression also occurs in EBV-associated cases with no amplification, suggesting that alternative mechanisms may induce PD-L1 expression in this molecular subgroup [40,44]. Overall, PD-L1 expression was observed in tumor and immune cells in up to 50% and 94%, respectively, of EBV-positive gastric carcinomas [44,45].

When clinical responses to Pembrolizumab therapy were analyzed according to TCGA molecular subtypes, patients harboring MSI-H (at present defined as MSI) and EBV-associated tumors achieved dramatic responses, as compared to genomically stable and chromosomal unstable subtypes [46].

The morphological features of gastric carcinoma with lymphoid stroma are also relevant to identifying these specific molecular subtypes, as EBV infection and MSI status are identified in up to 80% of cases [40,41,47]. Based on these findings, some authors have suggested systematically performing EBER *in situ* hybridization and immunohistochemistry for mis-

match repair proteins in gastric cancers presenting the morphologic features of gastric cancer with lymphoid stroma, for a cost-effective molecular characterization and selection of patients for targeted immunotherapies [40,48].

PD-L1 in small bowel adenocarcinoma

Small bowel adenocarcinoma (SBA) is a rare tumor, and, in virtue of this rarity, its treatment is hindered by a lack of robust data concerning treatment efficacy. Until recently, the National Comprehensive Cancer Network© (NCCN) guidelines suggested applying to SBA the same treatment protocol of colorectal cancer (CRC). However, SBA shares only limited similarities with CRC from a biological and molecular standpoint. In the same fashion, no strong and reliable data exist regarding PD-L1 in SBA, and its impact on both treatment and prognosis.

Variable percentages of PD-L1 positivity have been reported in the literature, ranging between 25% and 70% [49-54]. A study by Giuffrida et al. [51] reported higher percentages of PD-L1 positivity in SBA associated with celiac disease or Crohn's disease rather than in sporadic SBA (35% vs 35% vs 5%, respectively). In another study, Thota et al. [49] reported that SBA with pure mucinous histology or even the mucinous component of otherwise PD-L1-positive tumors did not show PD-L1 staining.

CPS is the most used score to evaluate PD-L1 expression [49-51,55] with values ≥ 1 considered positive; the ZEBRA trial [53], the largest multicenter phase II trial for Pembrolizumab use in advanced SBA, used the Modified Proportion Score (MPS), a score analogue to CPS, to evaluate PD-L1 in enrolled patients.

All these studies are characterized by relatively small study populations, owing to the rarity of the disease; moreover, some of these studies also include carcinomas of the ampulla of Vater in the group of SBA [55], while others only include non-ampullary duodenal carcinomas.

Regarding the impact of PD-L1 expression on prognosis, Klose et al. [50], showed a direct correlation between PD-L1 positivity and survival, in contrast with data from several other solid tumors. Giuffrida et al.,[51] reported that PD-L1 expression was associated with more favorable prognosis in univariate analysis, but no statistical significance was found in multivariate analysis.

Two large trials evaluated the efficacy of immunotherapy in SBA. The first one was KEYNOTE-158 [56] in 2019, which enrolled patients with MSI and non-colonic primary tumors. Nineteen patients with a diagnosis of SBA were enrolled, and the results showed a complete or partial response in approximately half of MSI SBA patients treated with Pembrolizumab (8/19; 42%).

The ZEBRA phase II trial [53] was a large multicenter trial for Pembrolizumab use in advanced SBA, whose results came out in 2021. The results of this trial did not report any complete response, and only three partial responses (3/40; 7.5%) were reported within the study population. Overall, these results were not satisfactory in the SBA unselected population but suggested that specific subgroups of SBA patients – MSI/dMMR and patients with a tumor mutation burden (TMB) > 10mt/Mb – may benefit from treatment.

Taking into account these results, Pembrolizumab and/or Nivolumab are currently recommended as a second-line treatment for MSI SBA patients [57].

PD-L1 in colorectal cancer

Although early studies had dismissed checkpoint inhibitors as an effective therapy in CRC, Pembrolizumab has been shown to give significant and durable responses in MSI CRCs both in pre-treated and in the naïve population by the KEYNOTE trials [58,59]. However, contrary to other solid cancers (*i.e.,* non-small cell lung cancer), PD-L1 positive immunostaining is not required for the patient to be eligible to receive the therapy; the value of PD-L1 as a biomarker has been evaluated only in the KEYNOTE-016 trial [60] and data on its predictive impact are very limited.

The use of MSI status as the stand-alone surrogate marker for eligibility for ICIs therapy is probably at the basis of the lack of standardization in the approach to PD-L1 evaluation in CRC. Different studies have used either the 1% or the 5% cut-offs to define positivity, while some studies have combined both intensity and percentage of positivity to grade PD-L1 immuno-reactivity, thus resulting in a plethora of papers that are scarcely comparable one to another in terms of results. This is also reflected by the variable percentage of reported PD-L1 positivity that can be found in the literature, which ranges from 9% [61] to 89% [62] on tumor cells and from 5% to 61% [62,63] on stromal cells.

There is also controversy whether MSS and MSI CRC differ in PD-L1 expression, with several studies reporting higher percentages (77-100%) of PD-L1 expression in MSI CRC [61,64,65], other studies reporting a statistically higher expression of PD-L1 in MSS CRCs [66], and other contributions reporting no significant differences whatsoever [67]. However, given that several characteristics are shared by MSI and PD-L1 CRCs (*i.e.,* right colon involvement, increased presence of tumor-infil-

trating lymphocytes [TILs], mucinous and medullary histology), we can infer that MSI tumors may be more represented among the PD-L1 positive CRCs. Indeed, expression of PD-L1 on CRC cells has been shown to correlate with the increased presence of CD8-positive TILs [61,66-69] and TBET-positive TILs [61]. FOXP3-positive T-regulatory lymphocytes (Tregs) number among TILs has been reported to be either increased [70] or decreased by tumor cell expression of PD-L1 [62].

PD-L1 expression has also been reported to be associated with poor tumor differentiation [61,63,67], higher T, N and M stages at diagnosis [63,67,71], higher tumor budding [67,72], epithelial-mesenchymal transition (EMT) [67], right-colon tumors [63,68] and mucinous [65,68] or medullary histology [61].

Furthermore, Rosenbaum et al. [61], reported a trend, albeit not statistically significant, toward *BRAF* mutations (a molecular alteration often found underlying MSI phenotype) being associated with PD-L1 positivity; it must be noted, however, that Omura et al. [71] reported inverse results, with *BRAF* mutations found more often in PD-L1-negative CRC. Most likely, the variance in these results can be attributed to different enrollment criteria and histological material (tissue microarrays vs whole-slide).

The prognostic impact of PD-L1 expression is controversial in CRC. PD-L1 expression is associated with a better prognosis in some studies [65,66], and with a worse one in others [61,73]. A direct impact on overall survival (OS) has not been consistently shown, but some studies have reported a positive correlation between PD-L1 expression and longer disease-free survival (DFS) [67,69]. A study by Wyss et al. [63] reported better OS and DFS in CRC showing stromal positivity for PD-L1, with no prognostic impact reported for tumor cell PD-L1 expression.

PD-L1 in anal squamous cell carcinoma

Anal squamous cell carcinoma (AnSCC) may express PD-L1 on tumor cells or tumor-related immune cells or both. PD-L1 expression by immune cells strongly correlates with CD8+ T cell density, suggesting a prevalent adaptive mechanism of PD-L1 expression driven by tumor-infiltrating lymphocytes in AnSCC [74]. However, constitutive (*i.e.*, in absence of significant intra-tumoral immune cell infiltration) and mixed patterns of expression have also been observed [75]. The PD-L1 positivity rate is wide, ranging from 22% to 85% and this is probably partly due to the fact that various scoring systems (TPS and CPS), positivity cut-offs, and antibody clones (E1L3N, 22C3, SP263, etc.) have been used in different studies. A recent study on 62 AnSCCs, using E1LN3 antibody, reported PD-L1 expression (CPS ≥ 1) in 32% of cases [76]. No difference in PD-L1 positivity rates has been noted between HIV+ and HIV-negative patients or between HPV+ and HPV-negative cases [74,76]. In the study by Monsrud et al.*,* HIV-positive patients with higher CD4 count were more likely to express PD-L1 on tumor tissue [77].

Also in this tumor, the impact of PD-L1 expression on prognosis is still debated. In some studies, PD-L1 (TPS or CPS) has been associated with worse OS, especially in HPV-negative AnSCCs [76-79], whereas in other papers PD-L1 positivity was associated with better survival [75,80,81].

A predictive role of PD-L1 in AnSCC has been suggested [82]. In phase II of the NCI9673 study, which was the first trial to establish the clinical benefits of immunotherapy in AnSCC, responders had higher PD-L1 expression on tumor cells [83], while in the phase II KEYNOTE-158 study treatment response was observed in 15% of AnSCC patients with CPS ≥ 1 (67% of cases) versus 7% of cases with CPS < 1 [56].

PD-L1 in pancreatic carcinoma

In pancreatic ductal adenocarcinoma (PDAC), the prognostic value of PD-L1 expression is still unclear and literature data about response to PD-1/PD-L1 inhibitors are not encouraging [84]. The peculiar milieu in PDAC, which is the continuous interaction between the glandular neoplastic component and tumor microenvironment (TME), could be the main factor affecting the poor response rate to ICI. In addition to cytokines and growth factors secreted by the TME promoting tumoral invasion, migration and angiogenesis, the intricate crosstalk between PDAC cells and TME also involves immune elements [85]. In PDAC, tumor-associated macrophages switch towards a M2 'immunosuppressive' phenotype, promoting tumor immunity and tumor progression; TILs produce high levels of PD-1 and interact with PDAC cells overexpressing PD-L1, resulting in T lymphocyte depletion. In this scenario, it is imperative to remember that the TME is an ever-changing non-static system [85]. Moreover, refractoriness of PDAC to PD-1/PD-L1 inhibition could be explained by technical issues such as the fact that the intensity and extent of PD-L1 staining were not taken into account when enrolling patients in clinical trials, by the use of at least four different diagnostic IHC assays, one for each of the currently available anti–PD-1/PD-L1 therapeutics, hindering the reproducibility and uniformity of testing and reporting results, and by the use of TPS instead

of CPS. Finally, preanalytical variables (sample collection, processing and storage), heterogeneity of expression of PD-L1 and adjunctive therapies may affect the results and interpretation of PD-L1 tests [86]. Interestingly, some differences in PD-L1 expression between neoplastic cells and immune cells have been found in pancreatic cancer; moreover, the prognostic value of PD-L1 differs according to tumoral histotype/molecular subtype. In the 'usual' PDAC, a positive PD-L1 expression rate ranging between 19% and 55%, correlation with poor tumor differentiation, more advanced tumor stage, and worse prognosis than PD-L1–negative PDAC are reported [86]. Enrichment for PD-L1 expression in frequency and extent, in comparison with conventional PDAC, was demonstrated in the undifferentiated histotype [86]; in adenosquamous histotype [87]; in sarcomatoid [88] and in anaplastic carcinoma with osteoclast-like giant cells [89]. In the latter subtype, PD-L1 expression has been associated with poor prognosis. No association was found between PD-L1 expression and Tumor Mutational Burden (TMB)/MSI profile in PDCA, suggesting an alternative pathway and expanding the pool of potential immunotherapy recipients [90]. Finally, PD-L1 mRNA is upregulated in squamous 'molecular' subtype *vs* other subtype in the Bailey's data set [91].

PD-L1 in biliary tract carcinoma

Assessing the prognostic and predictive value of PD-L1 in biliary-tract cancers (BTCs) is complex due to their heterogeneous nature. In fact, BTCs can be classified in intrahepatic, extrahepatic (hilar and distal) cholangiocarcinoma, and gallbladder cancer (GBC). These anatomical subtypes, moreover, have been further molecularly and genomically characterized demonstrating significant differences in mutations in *IDH1/2*, *FGFR2*, *PIK3CA*, *ERRB2*, *KRAS* and *BRAF* genes [92,93]. Several studies have tried to shed light on the complexity of the tumor microenvironment (TME) of BTCs. In fact, the immune system in BTCs is affected by chronic inflammation which plays an important role in their carcinogenesis [94]; moreover, CD8+ and CD4+ cells in neoplastic background have been demonstrated to be related with improved outcomes in BTC [95]. Conversely, data is available which shows that PD-1 expression in TILs contributes to an immunosuppressive environment [96].

As in pancreatic cancer, reproducibility and uniformity of results in different studies are affected by the different clones of PD-L1 used and by the use of TPS, CPS, and other scoring systems. Moreover, TME in BTC has been extensively investigated but the studies targeting PD-L1 have shown modest results in clinical activity in a small number of patients [94].

We selected the most recent studies performed on BTC samples dealing with PD-L1 expression and its relative used immunostaining scores.

Deng et al. [97] studied PD-L1 expression in tumor cells and TILs in 69 intrahepatic cholangiocarcinomas. Their results showed that the expression of PD-L1 in cancer cells, but not in TILs, was highly correlated with a poorer prognosis, suggesting that a 'high' level of expression of PD-L1 is associated with poor prognosis, and PD-L1 expression is a potential predictor and therapeutic target for intrahepatic cholangiocarcinoma.

Fontugne et al. [98] investigated PD-L1 expression in tumor cells and TILs in 41 perihilar and 58 intrahepatic cholangiocarcinomas. The authors showed a PD-L1 positivity rate of only 9% among all cases, although this increased to 46% when including PD-L1 positive surrounding inflammatory cells, suggesting a role for ICIs in therapy.

Gani et al. [99] examined PD-L1 in intrahepatic cholangiocarcinoma from 54 patients. Interestingly, in absence of PD-L1 expression in neoplastic cells, scoring was focused on tumor-associated macrophages (TAMs) and further subclassified in TAMs positive at the invasive tumor front (TF). The expression of PD-L1 within the TME, specifically within the TF, was found to be associated with an almost 60% shorter survival compared to patients whose tumors did not express PD-L1 in the tumor front.

Mody et al. studied the correlation between PD-L1 expression and molecular profiling in BTCs. PD-L1 positivity was found in 12% of GBC, 7% of intrahepatic, and 5% of extrahepatic cholangiocarcinoma. An increased frequency of PD-L1 expression was found in *BRCA2*, *KRAS*, and *BRAF* mutated BTC, suggesting the need study regimens combining specific targeted therapies with immunotherapies [94].

Concerning the relationship between PD-L1 and molecular features, Yoon et al. [100] proposed two resistance factors to PD-1/PD-L1 blockade in BTCs: *KRAS* alteration and Chromosomal Instability (CIN). According to their study, 95.0% of patients (19/20) having these factors did not show clinical benefit from anti-PD-1/PD-L1 agents in a PD-L1-positive cohort. Moreover, the authors directly demonstrated a suppressive immune TME with low TIL density in *KRAS*-altered or CIN tumors, suggesting that assessment of *KRAS* alteration and CIN status, combined with PD-L1 expression, could be a useful approach, and patient selection based on these factors may improve the efficacy of PD-1/PD-L1 blockade.

In conclusion, in the pancreatobiliary system, the role of PD-L1 expression in assessing the eligibility of pa-

tients for immunotherapy is limited and no agreement on PD-L1 clone and scoring system has been achieved.

Conclusions

A growing number of clinical trials are investigating the role of ICIs, alone or in combination, as therapeutic agents against several types of solid tumors, including gastrointestinal neoplasms. The use of PD-L1 IHC assays for stratification of patients and the identification of those who might benefit from ICIs is currently expanding and evolving. In this setting, the pathologist plays a central role in therapeutic decision-making. In fact, accurate biomarkers assessment is essential to ensure the best therapeutic option for the patient. PD-L1 evaluation does not come without challenges, due to the use of different companion diagnostic assays and scoring systems and the high levels of inter-assay variability. In the setting of gastrointestinal cancers, larger studies are needed to develop novel means of assessing PD-L1 expression over time and to establish the real prognostic and predictive value in bowel and pancreatobiliary neoplasms. Future perspectives include the use of digital pathology and automation, the incorporation of other biomarkers into the workflow to better reflect the tumor immune microenvironment, and the simplification of the regulatory landscape.

AUTHOR CONTRIBUTIONS

Conceptualization: MF, LM, AV; methodology, GP and FG; data curation, AV and PP; writing-original draft preparation, AV, PP, CR, IG, MLS, MC; writing-review and editing, AV, MF, LM, FG. All authors have read and agreed to the published version of the manuscript.

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