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Tissue- and liquid biopsy-based biomarkers for immunotherapy in breast cancer

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ABSTRACT

Immune checkpoint inhibitors (ICIs) have revolutionized cancer therapy and now represent the mainstay of treatment for many tumor types, including triple-negative breast cancer and two agnostic registrations. However, despite impressive durable responses suggestive of an even curative potential in some cases, most patients receiving ICIs do not derive a substantial benefit, highlighting the need for more precise patient selection and stratification. The identification of predictive biomarkers of response to ICIs may play a pivotal role in optimizing the therapeutic use of such compounds. In this Review, we describe the current landscape of tissue and blood biomarkers that could serve as predictive factors for ICI treatment in breast cancer.

The integration of these biomarkers in a "holistic" perspective aimed at developing comprehensive panels of multiple predictive factors will be a major step forward towards precision immune-oncology.

1. Introduction

A well-established strategy to reinvigorate endogenous immune response against tumors is the pharmacological blockade of immune checkpoint molecules such as the Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and the Programmed cell Death protein 1 (PD-1)/Programmed Death-Ligand 1 (PD-L1) axis using monoclonal antibodies (mAbs) named immune checkpoint inhibitors (ICIs). The employment of ICIs has revolutionized the field of cancer therapy and now represents the mainstay of treatment for many tumor types [1]. This applies also to breast cancer [2], where the blockade of PD-1/PD-L1 has entered clinical practice as a therapeutic option for both advanced- and early-stage triple-negative breast cancer (TNBC) [3], and several clinical trials are evaluating ICIs also in other breast cancer subtypes [4].

Both the anti-PD-L1 atezolizumab and the anti-PD-1 pembrolizumab have been approved in combination with chemotherapy as first-line therapy for patients with PD-L1-positive (PD-L1+) advanced-stage TNBC based on the results of the IMpassion130 [5,6] and KEYNOTE-355 [7,8] trials, respectively. Both trials showed a significant benefit in terms of objective response rate (ORR), progression-free survival (PFS) and overall survival (OS) from the addition of ICIs to chemotherapy in the PD-L1+ population -although significance for OS in IMpassion130 was not formally tested due to the hierarchical statistical design and the lack of significance in the intention-to-treat population.

However, in the IMpassion131 trial, which enrolled a similar population to that of IMpassion130 but employed paclitaxel instead of nab-paclitaxel as chemotherapy backbone, the addition of atezolizumab to chemotherapy did not lead to improvements in any of the endpoints [9]. Based on these findings, Roche, in consultation with the US Food and Drug Administration, decided in August 2021 to voluntarily withdraw the accelerated approval for atezolizumab in the USA, although this decision has no implication in Europe, where atezolizumab is still approved. The disappointing results of IMpassion131 well exemplify the concept that in cancer immunotherapy one size does not fit all, and the complexity of the biological processes driving the tumor–immune co-evolution is far to be fully elucidated [3].

Indeed, a substantial proportion of patients fail to respond to these therapies, either due to intrinsic or acquired resistance, revealing the

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implication of additional immunosuppressive pathways and suggesting the need for a precise selection of patients who would be more likely to benefit from such approaches [10].

As a matter of fact, the identification and validation of robust predictive biomarkers of response to ICIs may play a pivotal role in optimizing their therapeutic use. Moreover, since ICIs are not exempt from toxicity, a tailored use of such compounds can minimize the occurrence of potentially serious adverse events in the absence of significant clinical benefit. Overall, to fully unleash the therapeutic potential of ICIs and to ameliorate their risk-to-benefit and cost-effectiveness ratios, a bedside decision-making process based on available and innovative predictive biomarkers must be established. PD-L1 expression in the tumor is the most obvious biomarker for selecting patients who may benefit from ICIs. Nonetheless, many patients with PD-L1+ tumors actually do not benefit from ICIs and, on the other hand, PD-L1 negativity does not exclude the possibility of clinical benefit. Moreover, the clinical significance of PD-L1 positivity seems to be different across different disease settings. Indeed, other factors including both cancer cell-intrinsic and extrinsic features, such as tumor antigenicity, composition of infiltrating immune cells and spatial interactions between the various components of the tumor microenvironment may play an important role in determining sensitivity to ICIs. Here we review tissue and blood biomarkers that could serve as predictive factors for ICI treatment in breast cancer.

2. Tissue biomarkers

2.1. PD-L1 status

The assessment of PD-L1 expression in tumors by immunohistochemistry (IHC) assays is the most studied and applied biomarker for selecting patients to receive ICIs targeting the PD-1/PD-L1 axis [11].

In advanced TNBC, early phase I/II studies investigating ICIs monotherapy collectively showed that PD-L1 expression was associated with a higher probability of treatment benefit [12–15]. Moreover, among patients with PD-L1+ tumors, some evidence suggested an increasing probability of benefit with increasing PD-L1 expression levels [12,13,16]. This latter observation was confirmed also in KEYNOTE-119, the only randomized phase III trial to date comparing pembrolizumab monotherapy with chemotherapy in patients with previously treated mTNBC, irrespective of PD-L1 expression. The trial did not meet its primary endpoint of OS in patients with PD-L1 combined positivity score (CPS) $\geq$10, but an exploratory post-hoc analysis in patients with PD-L1 CPS $\geq$20 revealed an ORR of 26.3% vs 11.5% with chemotherapy and a numerical improvement of PFS and OS [17]. By contrast, the effect of chemotherapy on outcome appeared to be independent of PD-L1 expression.

The predictive value of PD-L1 was clearly demonstrated in the aforementioned IMpassion130 [5,6] and KEYNOTE-355 trials [7,8], in which the benefit derived from the addition of ICIs to chemotherapy was confined to patients with PD-L1+ tumors.

However, the implementation of PD-L1 assessment is far from being univocal and robust throughout different disease settings and ICI products, and the dichotomic classification of tumors based on PD-L1 status into either expressing or not expressing appears to be an oversimplification that does not account for the complex biological underpinnings of this biomarker [18]. Many lessons have been learnt from the extensive use of ICIs in other solid tumors.

First of all, PD-L1 can be expressed by both tumor cells and tumor-infiltrating immune cells, and different platforms (Ventana vs Dako) leveraging on different PD-L1-targeting mAb clones (e.g. SP142 for atezolizumab and 22C3 for pembrolizumab), do not show the same sensitivity and reproducibility in detecting PD-L1 expression on different cells [11]. Both Dako and Ventana platforms can provide the tumor proportional score (TPS) or tumor cell expression (TC), calculated as percentage of PD-L1-expressing tumor cells among all viable tumor cells; however, these scores do not find any direct application in ICIs therapy for TNBC.

In order to also account for PD-L1 expression on immune cells, the Ventana system can be used to generate the immune cell expression (IC) score, i.e. the percentage of tumor area infiltrated by PD-L1-expressing immune cells relative to the whole tumor area, while the Dako system provides the CPS as percentage of PD-L1-expressing tumor and immune cells relative to all (PD-L1+ and PD-L1-) viable tumor cells.

Besides different mAb clones, platforms and scoring algorithms, also the thresholds used to define positivity may be different ($\geq$10 for CPS vs $\geq$ 1% for IC), and each one of these aspects may influence the definition of PD-L1 positivity and, ultimately, impact on clinical indications (Fig. 1).

Additionally, several studies have also highlighted poor intra- and inter-assay concordance of companion diagnostic platforms in different tumor types [19–23], and some showed that both number and size of biopsy samples positively correlate with the likelihood of a proper and reproducible detection of PD-L1 expression [24].

In IMpassion130, the PD-L1 biomarker evaluated population (n = 614) was tested with both Ventana SP142 and Dako 22C3 mAbs, calculating the IC and CPS, respectively [25]. Only 73% of patients obtained concordant results (36% IC $\geq$ 1 and CPS $\geq$10; 37% IC < 1 and CPS <10), while 27% did not (10% IC $\geq$ 1 and CPS <10; 17% IC < 1 and CPS $\geq$10) (Fig. 1D). In the subgroup analysis, atezolizumab conferred no significant advantage in IC < 1 and CPS $\geq$10 TNBC, neither in terms of PFS nor OS [25]. This observation leads to the clinically meaningful observation that different PD-L1 IHC assessments should not be used interchangeably but, if available, both assays should be performed to tailor the most appropriate therapy to patients and avoid missing all potential candidates for ICIs therapy. Pragmatically, first-line treatment for TNBC with PD-L1 IC < 1 and CPS <10 should not include ICIs, tumors with IC $\geq$ 1 and CPS <10 should be treated with the combination of nab-paclitaxel and atezolizumab (where approved), and tumors with CPS $\geq$10 should be treated with the combination of chemotherapy and pembrolizumab (Fig. 1E).

Beside analytical variables, also biological factors should be taken in account for the correct appraisal of the predictive role of PD-L1. Tumors are naturally evolving and heterogeneous, both spatially and temporally, and several studies demonstrated that this heterogeneity is reflected also in PD-L1 expression [11,26–29]. For example, liver metastases usually present a lower lymphocytic infiltration and a higher likelihood of PD-L1 negativity, while the opposite is observed in lymph nodes [26,27]. Moreover, PD-L1 expression varies during therapy and different treatments may affect this dynamic in different ways [29] (Fig. 2).

The immunological differences between primary and metastatic breast cancer are well known [30,31]. Therefore, the different predictive significance of PD-L1 between the early and the advanced setting should not surprise [32]. The neoadjuvant KEYNOTE-173 [33], IMpassion031 [34], KEYNOTE-522 [35], GeparNuevo [36] and NeoTRIP [37] trials collectively showed that PD-L1+ tumors had a higher pCR rate than PD-L1- tumors irrespective of ICIs use, and the addition of ICIs to chemotherapy led to an increase in pCR rate compared to chemotherapy alone in both PD-L1+ and PD-L1- tumors [34–36], with NeoTRIP representing the only exception to this latter observation [37]. Notably, both KEYNOTE-522 [38] and GeparNuevo [39] demonstrated a statistically significant and clinically meaningful improvement in event-free survival in the immunotherapy arms without any significant difference across subgroups, including those defined according to PD-L1 expression, questioning the rightness of pCR as a surrogate endpoint to measure the role of ICIs in the neoadjuvant setting [40].

The diverse clinical significance of PD-L1 expression between early and metastatic setting and between different metastatic sites is consistent with the “seed and soil” theory and the notion that a tumor–immune co-evolution occurs during metastatic progression, moving from a more immune-activated microenvironment suitable to immunomodulation in early disease to a progressively increased immune-suppressed

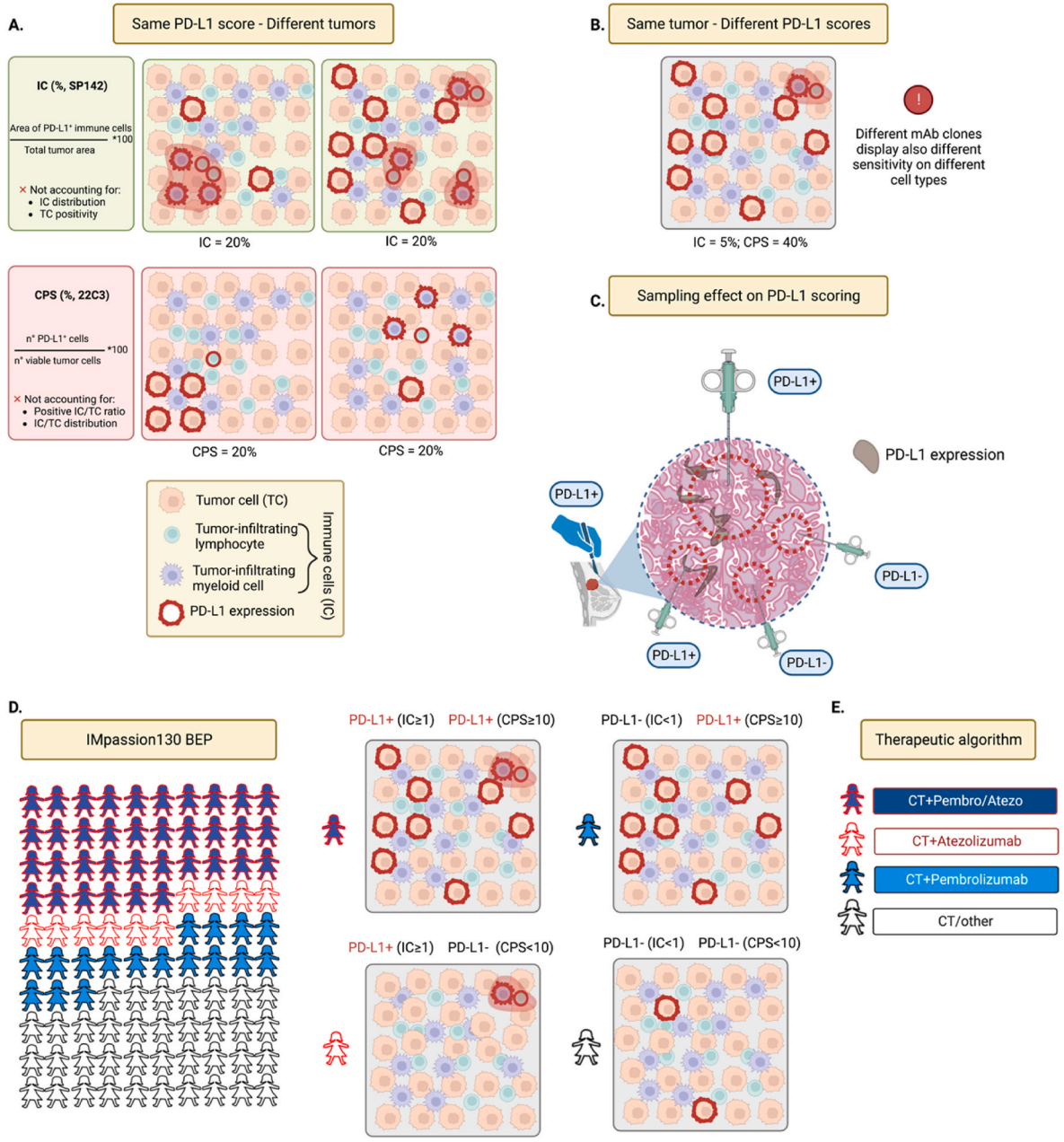

Fig. 1. Controversies of PD-L1 assessment. **A)** Graphical representation of different companion diagnostic PD-L1 scores for immune checkpoint treatment of advanced TNBC. The immune cell score (IC, Ventana platform, SP142 anti-PD-L1 clone), accounts for the percent total tumor area occupied by PD-L1 expressing immune cells. The combined positive score (CPS, Dako platform, 22C3 anti-PD-L1 clone), represents the percent ratio of PD-L1 expressing cells (both tumor and immune) on total viable tumor cells. IC does not account for immune cell distribution and PD-L1 expression on tumor cells. CPS lacks information regarding the cell types expressing PD-L1 and their spatial distribution. Both scores may return similar values despite significant biological differences in analyzed samples, as depicted. Graphical legend at the bottom; PD-L1 expression is represented as either red cell membrane or red shadows when considering cell numbers or tumor areas, respectively. **B)** Conceptual representation of intratumoural poor concordance of different PD-L1 scores. IC and CPS can provide different readouts on the same tumor sample, possibly affecting therapeutic decisions. **C)** Type, size, and number of tumor tissue sampling can impact PD-L1 detection. The wider the area and the higher the number of samplings, the greater the chances of PD-L1 positivity. Legend: surgical resection, surgeon hand and scalpel; Fine needle agobiopsy, small syringe; Core biopsy, big syringe; sampled areas, red-dotted area, IHC expression of PD-L1, brown areas. **D)** Comparison between PD-L1 assessment by SP142 (IC $\geq$ 1%) and 22C3 (CPS$\geq$10) in biomarker-evaluable population of IMpassion130. Overall percentage agreement is 73% (36% both positive; 37% both negative), while 27% of patients display opposite results with the two scores, highlighting the lack of interchangeability. Representative samples of each score combinations are shown on the right. **E)** Suggested therapeutic algorithm following the considerations from D. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phenotype in metastatic disease [3] (Fig. 2).

Overall, PD-L1 remains an imperfect predictor and several sources of variability may hamper its clinical utility as a standalone biomarker. Thus, for a more comprehensive view of the tumor–immune interactions, other biomarkers are needed and should be integrated with PD-L1 in order to shed light on the biological complexity beyond ICIs sensitivity.

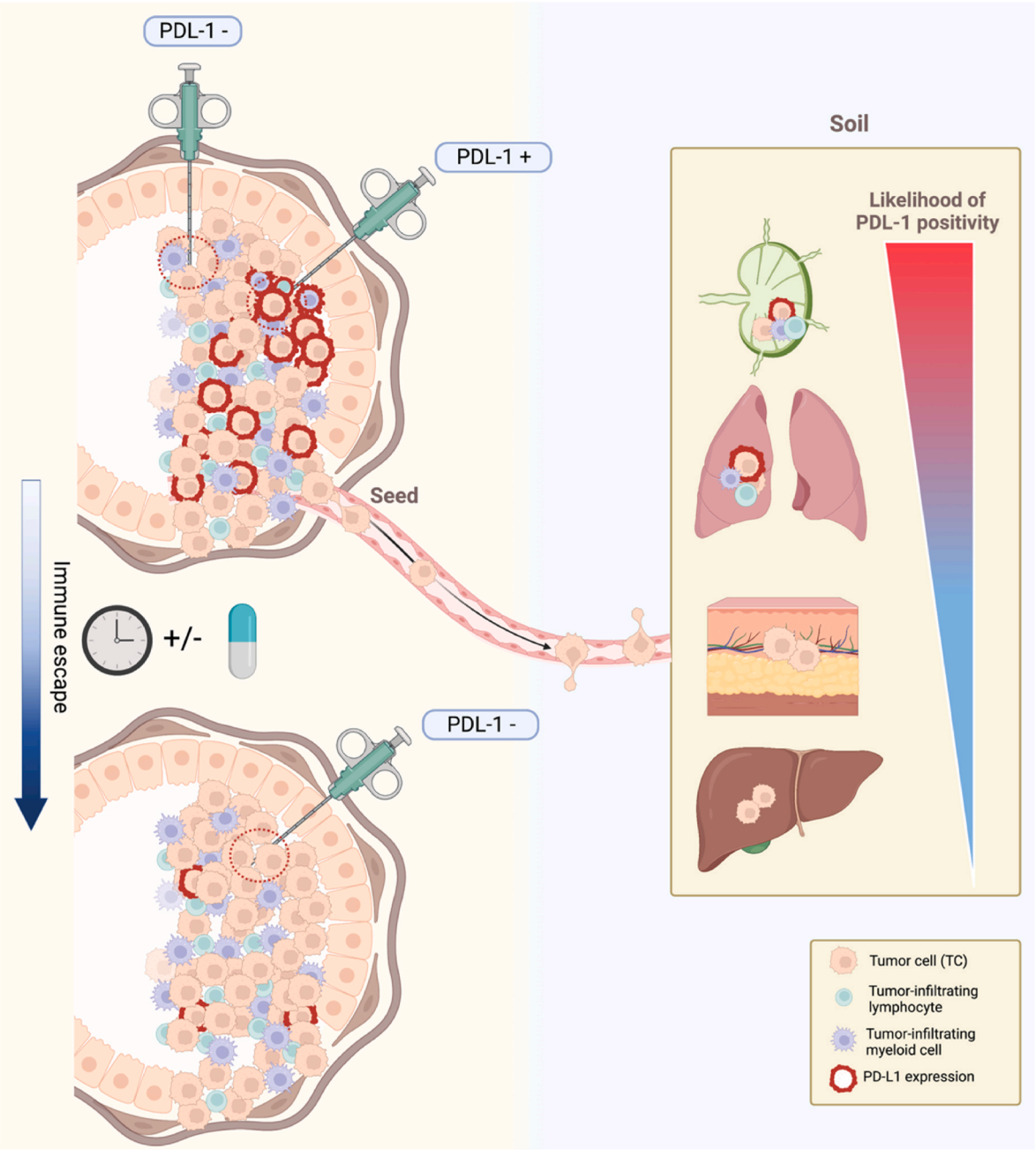

Fig. 2. Temporal and spatial heterogeneity of PD-L1 expression. Sources of PD-L1 assay variability through tissue biopsy encompass site of tissue sampling and dynamic changes in PD-L1 expression across the disease course. When clinically relevant, the concept of PD-L1 as a dynamic biomarker should be taken into account. Furthermore, for accurate PD-L1 detection in metastatic settings, it is crucial to consider the impact of the "soil" microenvironment in light of the tissue-specific likelihood of PD-L1 positivity.

2.2. *Tumor-infiltrating lymphocytes*

Tumor-infiltrating lymphocytes (TILs) are mononuclear immune cells that infiltrate the tumor tissue, and have been described in most types of solid tumors, including breast cancer [41]. In breast cancer the majority of TILs are typically located in the tumor stroma (sTILs) and are represented by a lymphocyte population mainly comprised of cytotoxic $CD8^+$ T cells, together with varying proportions of helper $CD4^+$ T cells, $CD19^+$ B cells, and NK cells [42,43]. The prevalence and clinical significance of TILs are different across breast cancer subtypes. In TNBC and HER2+ breast cancer TILs are more abundant compared to luminal tumors, and higher levels of TILs are associated with a more favorable outcome [44,45].

Early studies investigating ICI monotherapy in metastatic TNBC have shown that the clinical activity of both pembrolizumab and atezolizumab was highest in patients with $CD8^+$ T cells- and/or sTILs-positive tumors [46,47]. In the phase I PCD4989g trial, improved ORRs (14% vs 6%) and longer PFS (HR 0.69 [0.44–0.98]) and OS (HR 0.61 [0.39–0.96]) were observed in patients with higher (above median) baseline $CD8^+$ T-cells treated with atezolizumab monotherapy [46].

In the phase II KEYNOTE-086 study, investigating pembrolizumab monotherapy in pretreated PD-L1+/− (cohort A) and in untreated PD-L1+ (cohort B) metastatic TNBC, median TIL levels were higher in responders vs nonresponders (10% vs 5% in cohort A; 50% vs 15% in cohort B) [47]. In patients with TIL levels above vs below median, ORR was 6% vs 2% in cohort A and 39% vs 9% in cohort B. In this study, TILs

significantly correlated with PD-L1 expression assessed by CPS but were independent predictors of response to pembrolizumab [47].

The KEYNOTE-119 trial corroborated the association between higher TILs and greater benefit from ICI monotherapy [48]. In this trial, TILs levels were significantly higher in responders vs nonresponders in the pembrolizumab but not in the chemotherapy arm and were significantly associated with ORR (p = 0.0004), PFS (p = 0.0002) and OS (p = 0.0003) only in the pembrolizumab arm. Median OS in the pembrolizumab and chemotherapy arm were 5.9 and 8.8 months for patients with TILs <5% (HR 1.50 [95% CI, 1.14–1.97]), and 12.5 and 11.3 months for patients with TILs ≥5% (HR 0.75 [95% CI, 0.59–0.96]), respectively. The correlation between TILs and CPS was moderate and these two biomarkers showed independent predictive value in the multivariate analysis [48].

In the IMpassion130 study, the median value of sTILs was 5% and sTIL levels were moderately correlated with PD-L1 as a continuous variable. Using a prespecified threshold of 10% or more to define sTIL positivity, patients with sTILs + tumors in the atezolizumab arm showed longer PFS and OS than those in the placebo arm. However, patients with sTILs + tumors benefited from the addition of atezolizumab to nab-paclitaxel only if their tumors were also PD-L1+ (HR for PFS: 0.54 [0.39–0.75]; HR for OS: 0.64 [0.43 to 0.96]), while no benefit was observed compared to placebo in patients with sTILs+ and PD-L1- tumors (HR for PFS: 0.92 [0.59–1.44]; HR for OS: 1.04 [0.59–1.82]), suggesting that sTILs did not provide an additional predictive value for atezolizumab benefit beyond PD-L1 [27].

The substantial contribution of the host immune response to the therapeutic effects of HER2-directed monoclonal antibodies has provided a strong rationale for the investigation of immunotherapy in HER2-positive breast cancer [49]. In this context, a correlation between TILs and ICIs benefit has been shown in two separate studies [50]. In the phase Ib-II PANACEA trial investigating pembrolizumab plus trastuzumab in trastuzumab-resistant patients, TILs levels were significantly higher in responders vs nonresponders (p = 0.006) and in patients achieving disease control vs those with progressive disease (p = 0.0006) [51]. Similarly, in the phase II randomized KATE2 trial evaluating the addition of atezolizumab or placebo to TDM-1 in previously treated HER2+ metastatic breast cancer, higher TIL levels were associated with PD-L1 positivity and the addition of atezolizumab to TDM-1 was associated with a lower risk of disease progression only in patients with high TILs (HR for PFS: 0.62 [0.37–1.03] in patients with TILs ≥5% vs 1.52 [0.76–3.04] in patients with TILs <5%) [52].

The predictive role of TILs in the context of ICIs treatment has been investigated also in early-stage disease. Data about TILs in KEYNOTE-522 [35] and IMpassion031 [34] trials are eagerly awaited, while evidences from the KEYNOTE-173, GeparNuevo and NeoTRIP trials indicated that pre-treatment sTILs were significantly associated with pCR after neoadjuvant treatment with ICIs plus chemotherapy [29,33,36]. However, in early-stage TNBC higher levels of TILs were associated with a higher likelihood of achieving a pCR also after standard neo-/adjuvant chemotherapy [53–56], and the results from the control arms of GeparNuevo and NeoTRIP corroborated this association. Therefore, similar to PD-L1, the use of TILs to select patients with TNBC for treatment with ICIs in the early setting is not straightforward.

Emerging evidence suggests that the specific immune cell composition and spatial distribution in the tumor microenvironment can be more informative than the simple quantification of infiltrating immune cells [57,58]. For example, data from the I-SPY 2 trial showed that the macrophage to cytotoxic T cell ratio was negatively associated with response to neoadjuvant pembrolizumab plus chemotherapy, and that the spatial distribution of $CD3^+$ T cells in proximity to cancer cells positively correlated with pCR [59].

In the NeoTRIP trial, we showed that high density of PD-L1+/IDO + antigen presenting cells and of $CD56^+$ neuroendocrine epithelial cells are positively associated with pCR after atezolizumab plus chemotherapy but not after chemotherapy alone (p for interaction = 0.004) [60]. Moreover, high degree of spatial connectivity between epithelial cells and specific cell phenotypes of the tumor microenvironment (e.g. CD8+/PD1+ exhausted T cells; CD8+/granzyme B + T cells; $CD20^+$ B cells) was predictive of higher pCR rates with atezolizumab (but not with chemotherapy alone), independent of PD-L1 expression and sTIL levels [60].

Finally, the pharmacodynamic modulation of specific immune cell populations during and after ICIs treatment may add relevant predictive information for treatment benefit. In GeparNuevo, an increase of TILs in post-window samples compared with pre-treatment samples was predictive of pCR in the durvalumab arm (OR 9.36, p = 0.029) but not in the placebo arm [36]. In NeoTRIP high sTILs (≥40%) after 1 cycle of treatment showed a stronger correlation with pCR (OR 6.87, p = 0.0007) than baseline sTILs [29].

The dynamic monitoring of specific immune cell subsets during ICI therapy may also shed light on the biological mechanisms underlying treatment responses in the metastatic setting [61,62]. However, further studies are needed to better characterize the contribution of TILs dynamics in determining tumor responsiveness and patients' outcome.

2.3. Tumor mutational burden

Somatic mutations occurring in tumor DNA are the main source of tumor-specific neoantigens which are a key target of anti-tumor immune response [63,64]. As the immunogenicity of tumor neoantigens is stochastically determined, it is reasonable to assume that the higher the number of non-synonymous mutations in a tumor, the higher the odds of generating an effective antitumor immune response after inhibition of checkpoint signals [65,66]. Indeed, tumor mutational burden (TMB), defined as the number of somatic mutations per mega-base (mut/Mb) arising in tumor-coding regions [67], is associated with neoantigen load, T-cell infiltration and expression of immune gene signatures, and accumulating evidence suggests that a high TMB may be predictive of response to ICIs across several tumor types [68,69].

This evidence led to the FDA agnostic approval of pembrolizumab for patients with metastatic TMB-high (defined as ≥10 mut/Mb) solid tumors, based on results from phase II KEYNOTE-158 trial [70]. However, breast cancer was not included in this study.

Compared to other cancers in which immunotherapy has been established for a longer time such as lung cancer or melanoma, breast cancers generally carry lower TMBs [71]. In a large study using publicly available data from 3969 patients with primary or metastatic breast cancer, the overall median TMB was of 2.63 mut/MB and only 5% of all cases were ranked as TMB-high by applying the common definition of ≥10 mut/MB [72].

In the early setting, the predictive role of TMB has not been extensively investigated. Data from the GeparNuevo trial suggested that TMB is predictive of pCR in patients with TNBC treated with neoadjuvant chemotherapy with or without immunotherapy (OR for pCR per mut/MB = 2.06 [1.33–3.20], p = 0.001). However, albeit evident in both arms, the association between pCR and TMB seemed to be stronger in the chemotherapy alone arm (OR 2.82 [1.21–6.54], p = 0.016) than in the durvalumab plus chemotherapy arm (OR 1.77 [1.00–3.13], p = 0.049) [73]. In this trial, despite a modest and not significant increase in pCR rates (from 44% to 53%, OR 1.45; p = 0.287), the addition of durvalumab to neoadjuvant chemotherapy significantly improved both distant disease-free survival (DDFS) and OS at 3 years [39]. Interestingly, an exploratory survival analysis in patients stratified according to TMB dichotomized using the upper tertile of the cohort showed that only patients with low TMB seemed to benefit from the addition of durvalumab to neoadjuvant chemotherapy (HR for DDFS = 0.23 [0.06–0.70], p = 0.02), while no differences were detected in patients with high TMB (HR = 0.95 [0.19–4.69], p = 0.95) [74].

In contrast, in the metastatic setting, current evidence suggests a positive correlation between high TMB and benefit from ICIs. An exploratory analysis of KEYNOTE-086 showed that TMB was

significantly associated with ORR, PFS, and OS after adjustment for PD-L1, T-cell-inflamed gene expression profile or sTILs [16]. In IMpassion130, there was no correlation between TMB and PD-L1 status, but increasing TMB was associated with improved PFS and OS in the atezolizumab arm only in patients with PD-L1 + tumors (highest TMB quartile: HR for PFS = 0.31 [0.17–0.57]; HR for OS = 0.37 [0.15–0.90]), while no correlation between TMB and outcome was detected in patients with PD-L1– tumors [75].

In KEYNOTE-119, a potential association between TMB and clinical benefit was observed with pembrolizumab (p = 0.014 for PFS and 0.018 for OS) but not with chemotherapy (p = 0.478 for PFS and 0.906 for OS) [76]. Although limited by the small sample size (n = 26), a trend toward increased benefit with pembrolizumab vs chemotherapy was shown in patients with TMB ≥10 mut/MB (ORR 14.3% vs 8.3%; HR for OS = 0.58 [0.21–1.57]), but not in patients with TMB <10 mut/MB (n = 227, ORR 12.7% vs 12.8%; HR for OS = 0.81 [0.61–1.07]) [76].

The encouraging findings supporting a potential predictive role of TMB for ICIs provided the rationale for the design of the nonrandomized phase II TAPUR trial investigating single-agent pembrolizumab in patients with metastatic breast cancer of any subtype and high TMB (defined as TMB ≥9 mut/MB). In this cohort of heavily pre-treated patients (26 out of 28 patients with 3 or more prior systemic therapies) ORR was 21% (95% CI 8–41%), median PFS 10.6 weeks (95% CI 7.7–21.1 weeks) and median OS 30.6 weeks (95% CI, 18.3–103.3 weeks). However, no association between increasing TMB and longer PFS was found [77].

In line with these data, a retrospective analysis of 62 patients with metastatic TNBC treated with ICI mono- or combination therapy showed that patients with TMB-high tumors derived a significantly larger benefit from ICIs than patients with low TMB (OR of response = 4.32, p = 0.05; mPFS = 12.5 vs 3.7 months, p = 0.03; mOS 29.2 vs 14.2 months, p = 0.06). Again, the association between TMB and outcome was independent of PD-L1 status [78].

More recently, the single arm, phase II NIMBUS trial evaluated the efficacy of the combination of nivolumab plus ipilimumab in 30 patients with TMB-high (TMB ≥9 Mut/Mb) HER2-negative metastatic breast cancer. ORR was 16.7% and was not significantly different according to PD-L1 status and TIL levels [79]. Interestingly, patients with TMB ≥14 mut/Mb derived a much larger benefit from therapy than patients with TMB <14 mut/Mb (ORR 60% vs 8%,p = 0.02; mPFS 9.5 vs 1.4 months, HR 0.3 [0.08–1.06]; and mOS not reached vs 8.9 months, HR 0.2 [0.03–1.9]), suggesting that the optimal TMB cutoff for prediction of ICI benefit in breast cancer has not yet been well defined [79].

Collectively, these data indicate that the predictive role of TMB for ICIs benefit is far to be established, and this might be explained by both methodological and biological limitations of this biomarker. Indeed, the TMB measurement methods are not yet standardized, and different studies have used different platforms for its calculation. Several experimental factors, including sequencing depth and read length, variant calling algorithms and filters used to remove germline variants, can significantly affect TMB values [80]. Moreover, an increasing amount of evidence suggests that not all mutations are equally immunogenic and that therefore mutation quality may be even more important than mutation quantity [80–82]. The predictive value of the TMB could be improved by considering mutation quality or the presence of specific mutational signatures (such as the APOBEC signature [83]). Such issues highlight the limitations of implementing TMB alone to guide immunotherapy choices in breast cancer.

2.4. Gene expression signatures

A large amount of evidence supports the notion that a pre-existing anti-tumor immune response is essential -albeit not sufficient- for the efficacy of PD-1-/PD-L1–directed therapies [84]. Indeed, several studies showed that immune gene signatures reflecting an inflamed tumor microenvironment characterized by IFN-γ signaling, cytotoxic effector cells, active antigen presentation, and T cell cytokines may have a predictive role for response to ICI therapy.

One of the most well described gene signatures was the T cell-inflamed gene expression profile (GEP), which contained IFN-γ–responsive genes related to antigen presentation, chemokine expression, cytotoxic activity and was associated with clinical benefit from pembrolizumab across several cancer types, including breast cancer [85,86]. The predictive role of T cell–inflamed GEP was evaluated in patients enrolled in the KEYNOTE-086 trial and showed a statistically significant association with both PFS and OS (P < 0.001) [87]. In this trial, another signature using 37 tissue-resident memory T cell-related genes was also significantly associated with response to pembrolizumab but was highly correlated with T cell–inflamed GEP and did not add independent information [87]. Of note, T cell–inflamed GEP may provide predictive information for pembrolizumab benefit independent and complementary to that provided by TMB [88].

Another gene signature that has been shown to provide significant predictive information for ICIs benefit was the 27-gene IO-score signature [89]. This signature was evaluated in a cohort of TNBC patients enrolled in a phase I/II trial of neoadjuvant chemo-immunotherapy and showed a statistically significant predictive power for pCR with durvalumab (OR 4.13, p = 0.012) [90]. In the NeoTRIP trial, we showed that IO-score was significantly predictive of pCR in the atezolizumab plus chemotherapy arm (OR 3.64 [1.68–7.90], p = 0.001), but not in the chemotherapy alone arm (OR 1.31 [0.64–2.67] (p = 0.46) (test of interaction p = 0.029) [91]. Moreover, we also found that an early on-treatment evaluation of IO-score may add significant predictive information for pCR with atezolizumab, with assessment after 1 cycle of treatment being more informative than that at baseline. The combination of baseline and on-treatment binary IO-score defined four groups with significantly different likelihood of pCR (73.7% in positive/positive vs 15.2% in negative/negative groups, OR = 15.68, p = 0.0001), suggesting that the dynamic of IO-score may represent an early surrogate of ICIs benefit [92].

The predictive performance of immune gene signatures for ICIs response was also assessed in the GeparNuevo trial [93]. Two signatures were evaluated in this study: the GeparSixto signature - a set of immune genes that has been shown to predict response to neoadjuvant chemotherapy in triple-negative and HER2-positive breast cancer [94], and the IFNγ-signature described by Higgs and colleagues as predictive for response to durvalumab in lung and urothelial cancer [95]. Both were associated with increased pCR rates but without specificity for durvalumab. In an exploratory analysis, the authors identified seven genes related to antigen presentation and IFN signaling (HLA-A, HLA-B, TAP1, GBP1, CXCL10, STAT1, and CD38) that were significantly associated with pCR in the durvalumab arm, but not in the placebo arm [93].

In the I-SPY 2 trial, a set of 53 immune-related genes named ImPrint has been shown to predict pCR to pembrolizumab with overall sensitivity >90% and specificity >80%. Importantly, the signature appeared to be predictive for ICIs benefit also in HR + HER2-patients (sensitivity >80%, specificity >85%) [96]. Regarding TNBC, different gene expression signatures reflecting immune activation (n = 8) showed a strong correlation with response to pembrolizumab and, among them, dendritic cells and STAT1_sig/chemokine12 gene signatures were the most predictive [97]. Overall, patients with TNBC expressing the immune signatures (Immune +) had a pCR rate of 89% compared to 27% in patients without signatures expression (p = 0.0013). Moreover, gene expression signatures related to DNA repair deficiency (DRD) added further predictive information for pembrolizumab benefit (pCR 92% in Immune +/DRD + vs 20% in Immune -/DRD -) [97].

Several studies in solid tumors have shown an association between the presence of T cells displaying residency properties in the tumor microenvironment and improved outcome [87,98–100]. Recently, Virassamy and colleagues characterized the critical role of tissue-resident memory (TRM) T cells in mouse models of TNBC and derived a TRM-specific gene signature which was subsequently tested in the I-SPY

and GeparNuevo datasets [101]. In I-SPY, higher signature expression was associated with a higher chance of achieving a pCR in both ER+/HER2-negative breast cancers and TNBC regardless of treatment, but in TNBC this association only held for patients treated with pembrolizumab. In GeparNuevo, the signature was associated with higher pCR rates and, more importantly, with excellent survival outcomes in the durvalumab arm but not in the placebo arm ($p = 0.0051$ for DDFS, $p = 0.0052$ for OS) [101].

The integration of gene expression profiling with the quantification and spatial distribution of immune cells has led to the identification of four tumor immune microenvironments (TIME), namely "immune desert" and "fully inflamed", with homogeneously low or high numbers of $CD8^+$ T cells, respectively, and "margin restricted" and "stroma restricted", with compartmentalized $CD8^+$ T cells in the tumor margins or stroma, respectively [57]. The association between TIME subtype and outcome has been investigated in a retrospective analysis of the IMpassion130 trial, which showed that the fully inflamed subtype was linked to improved PFS and OS with atezolizumab and nab-paclitaxel in PD-L1+ tumors [102]. Furthermore, an increased expression of genes related to proliferation and DNA repair pathways has also been associated with improved PFS, while increased angiogenesis, EMT, hedgehog signaling, estrogen response, and TNF signaling pathways have been associated with treatment resistance [102].

Many other gene signatures have been identified as predictors of ICIs benefit in other malignancies [103–106], but their role in breast cancer remains to be fully elucidated.

3. Blood biomarkers

Tissue biopsy is still considered the gold standard to molecularly characterize a tumor and identify prognostic and predictive biomarkers useful to drive treatment decisions, and this also applies to immunotherapy. However, tissue biopsies are not able to fully capture spatial and temporal tumor heterogeneity, and less-invasive and more cost-effective techniques are needed to overcome such limitation [107]. In this scenario, liquid biopsies assessing blood biomarkers could help to more comprehensively address the heterogeneity of metastatic cancer and characterize the systemic immune status associated with ICIs response or resistance [108,109].

3.1. Lactate dehydrogenase

Lactate dehydrogenase (LDH) is one of the most studied blood biomarkers in cancer and has been historically considered a marker of poor prognosis [110], primarily due to its correlation with tumor burden. Besides its prognostic significance, increasing evidence suggests a role of LDH in the modulation of several biological processes, including anti-tumor immune response [111]. Indeed, high LDH levels may impair T cell functionality and proliferation, increase NK cells apoptosis and sustain Treg suppressor functions [111]. Consistently with evidences in other solid tumors, higher LDH levels were associated with lower ORR and shorter survival with ICIs monotherapy in patients with pretreated mTNBC [13,14,46]. However, the predictive role of LDH levels in patients treated with ICI combinations is less clear.

3.2. CD163

The hemoglobin scavenger receptor CD163 is a macrophage-specific marker upregulated by anti-inflammatory cytokines and mainly associated with M2 polarization [112]. The soluble variant of CD163 (sCD163) is constitutively present in plasma and high levels of sCD163 have been significantly associated with poor OS in patients with several cancer types [113]. Moreover, serum levels of sCD163 increased significantly in melanoma patients responding to nivolumab [114], suggesting a potential predictive role of sCD163 dynamics. Indeed, biomarker analysis of a phase II trial investigating the combination of nivolumab, paclitaxel and bevacizumab in patients with HER2-negative metastatic breast cancer revealed that patients with increased sCD163 after 1 week of treatment had significantly longer PFS compared to patients with sCD163 decrease (mPFS 18.2 vs 13.6 months, HR 0.50 [0.26–0.93], $p = 0.0263$), with, although not statistically significant, a similar trend in OS ($p = 0.0548$) [115].

3.3. Circulating tumor DNA

Circulating tumor DNA (ctDNA) analysis is a powerful research tool that can be used for several purposes in cancer, such as early detection, patient prognostication, longitudinal monitoring of treatment response and selection of mutation-directed therapies [108]. However, limited data exist about the prognostic and predictive role of ctDNA in patients receiving ICIs. In a prospective phase II trial assessing ctDNA dynamics in patients with advanced solid tumors (including TNBC) treated with pembrolizumab, Bratman and colleagues showed that baseline ctDNA levels were significantly associated with survival (lower-vs higher than-median ctDNA: HR for PFS 0.54 [0.34–0.85]; HR for OS 0.49 [0.29–0.83]), and that on-treatment ctDNA dynamics were even more informative for outcome prediction (decrease vs increase from baseline: HR for PFS 0.33 [0.19–0.58]; HR for OS 0.36 [0.18–0.71]) [116].

In the I-SPY2 trial, ctDNA clearance after 3 weeks of treatment was significantly associated with pCR ($OR = 1.92$, $p < 0.001$) and all patients who achieved pCR had no ctDNA detectable before surgery. Additionally, among patients who failed to achieve pCR, distant recurrence-free survival was significantly better in those with complete ctDNA clearance (HR 0.13; 95% CI 0.05–0.37) [117].

Genetic analysis of ctDNA may also allow for the evaluation of TMB with liquid biopsy, and some data in lung cancer suggest that high blood TMB can be predictive of benefit from immunotherapy [118]. In breast cancer, preliminary evidence suggests that a decrease in variant allele frequency of ctDNA mutations during treatment may represent a marker of response to immunotherapy [119].

3.4. Circulating tumor cells

Beyond secreted factors, studies have also examined whether the repertoire of specific cell populations in blood, including circulating tumor cells (CTCs), may provide prognostic information for ICIs benefit.

Given all the above-mentioned limitations of tissue-based PD-L1 assessment, the evaluation of PD-L1 expression on CTCs has aroused some interest in clinical research in different tumor types, including breast cancer [120,121]. Despite some conflicting results, current evidence suggests that the reduction of PD-L1+ CTCs in patients treated with ICIs may correlate with response while, on the contrary, their persistence during treatment has been associated with worse prognosis [122]. However, these studies included only few patients with breast cancer and therefore further work is needed to better elucidate the prognostic and/or predictive role of PD-L1 expression on CTCs in the context of ICI therapy.

3.5. Circulating T cells

PD-L1 expression can be found also in circulating immune cells. An exploratory analysis of the GeparNuevo trial evaluating the changes of immune cell repertoires in the blood during and after neoadjuvant treatment, revealed that the administration of durvalumab resulted in an almost complete loss of detectable PD-L1+ $CD4^+$ and $CD8^+$ T cells, while no effect on these cell populations was evident with placebo (*t*-test $p < 0.05$) [123]. Moreover, both baseline and on-treatment assessment of different immune cell types have been shown to be informative of treatment effects. For example, higher levels of $CD4^+$ T cells at baseline and the expansion of γδ T cells during treatment correlated with better response to durvalumab and chemotherapy, but not to chemotherapy alone [123].

3.6. Eosinophils

Besides T lymphocytes, many other immune cell populations are implicated in anti-tumor immunity. Among these, eosinophils have been demonstrated to exert pleiotropic functions with both anti- and pro-tumorigenic significance [124]. In the context of ICIs treatment, the available data collectively suggest that eosinophils may be capable of boosting tumor immunity through both direct and indirect mechanisms, thus providing a relevant contribution to the response to cancer immunotherapy [125]. Eosinophilia has been reported to correlate with positive outcomes following ICI therapy in many tumors, including breast cancer [126]. In a phase I/II trial testing the combination of durvalumab and weekly paclitaxel in patients with mTNBC [127], an increased blood eosinophil count during treatment was significantly associated with PFS ($p = 0.005$), and a similar trend, although not statistically significant ($p = 0.167$), has been observed also for OS [126]. More recently, a longitudinal analysis of fresh blood and tumor biopsy samples obtained from mTNBC patients enrolled in the TONIC trial revealed that circulating eosinophils significantly increased in patients responding to nivolumab and that the increased expression of an eosinophil gene signature correlated with increased $CD8^+$ T cell and IFN-g gene signatures in responders, suggesting that eosinophils may provide an active contribution to ICI response [128]. More importantly, patients with increased circulating eosinophils had longer PFS and OS [128].

3.7. Neutrophil to lymphocyte ratio

Another extensively studied cellular blood biomarker is the neutrophil to lymphocyte ratio (NLR). NLR may reflect the dynamic

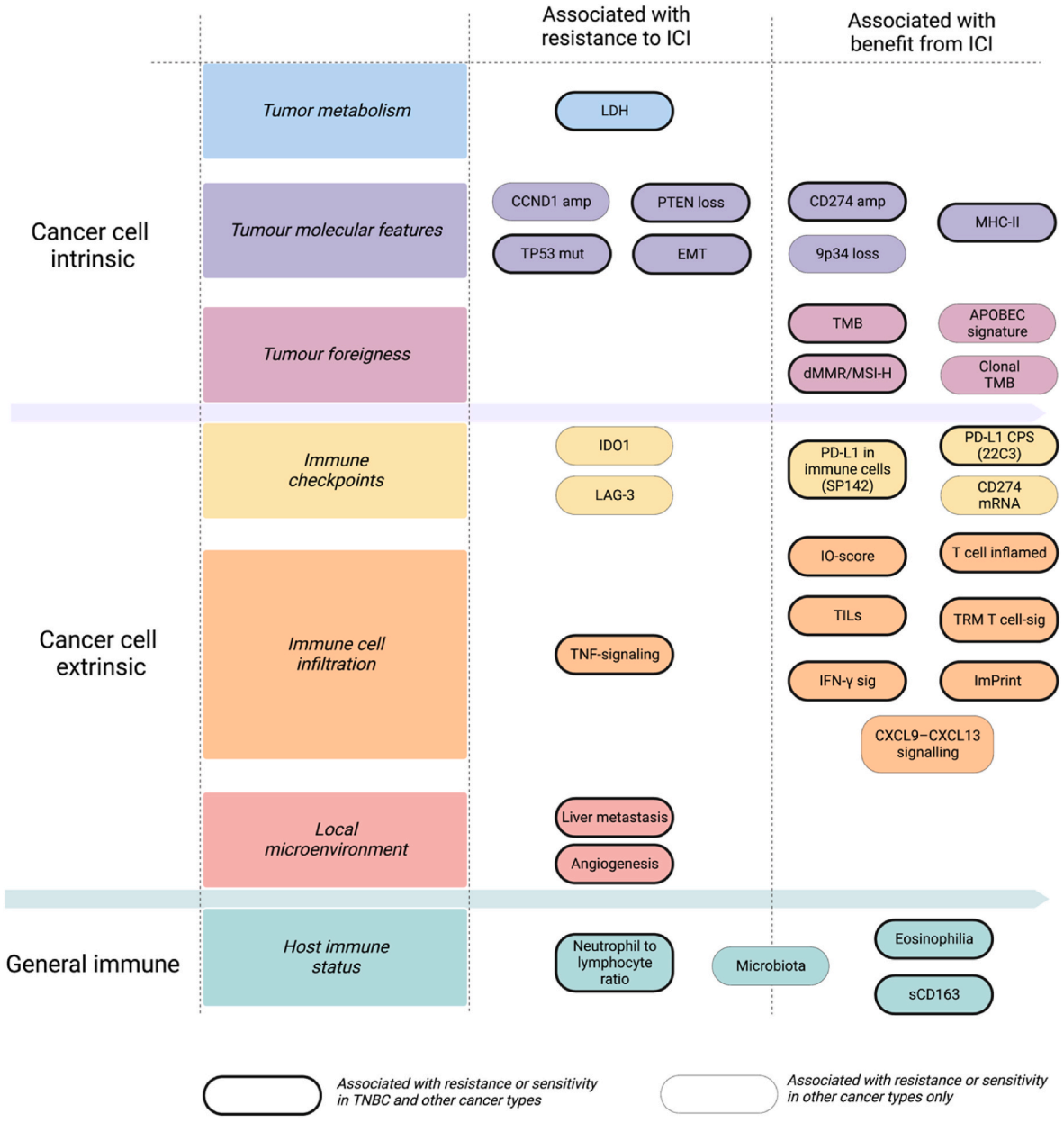

Fig. 3. Landscape of biological features associated with immunotherapy response. Cancer cell intrinsic and extrinsic features, including host immunity, impact the effectiveness of immune checkpoint inhibitors targeting the PD-1/PD-L1 axis. The features reported are associated to resistance or sensitivity in either TNBC and other cancer types (bold contoured boxes) or exclusively in other cancer types (thin contoured boxes). Comprehensive assessment of such factors may be pivotal to select patients that are more likely to respond to ICI. (Adapted and updated from Bianchini et al. Nat Rev Clin Oncol 2022).

equilibrium between anti-tumor immune response and pro-tumorigenic inflammation [129], and several studies in different tumor types have shown that high NLR is an independent predictor of worse prognosis [130–132]. The clinical significance of NLR in the context of ICIs has been mainly investigated in melanoma, lung cancer and renal carcinoma [133–135], while data in breast cancer are scarce. In a retrospective analysis of 1714 patients with 16 different cancer types treated with ICIs, Valero and colleagues showed that higher NLR was associated with poorer OS and PFS [136]. However, in the breast cancer cohort this association did not reach statistical significance, probably due to the small sample size (n = 27) [136].

Taken together, the data presented above highlight the potential of liquid biopsy-based biomarkers to become useful tools in the management of breast cancer patients treated with ICIs. Yet, despite not being ready for prime time, the ever-growing technological advances in this field foresee an implementation of liquid biopsy in clinical practice in the next future.

4. Additional potential biomarkers

The mechanisms of action of ICIs are, at least partially, agnostic from tumor histology. Therefore, it is not surprising that several predictors of ICIs response have demonstrated a pan-cancer significance. Among these, tumor- and T cell-intrinsic biomarkers recognized also in breast cancer include, but are not limited to, *CD274* (the gene encoding PD-L1) gain or amplification [137], 9q34 (TRAF2) loss [83], *BRCA2* deficiency [138], *POLE/POLD1* mutations [139], *CCND1* amplification [83], CXCL9 expression [83] and MHC-II expression on tumor cells [60,140] (Fig. 3). Regarding *CD274* gain or amplification, it is worthy to note that in the SAFIR02-BREAST IMMUNO trial, this genomic alteration was associated with increased PD-L1 expression in cancer cells but not in immune cells, suggesting a possible cancer cell-specific expression pattern [137]. However, further studies are needed to validate the role of these factors as predictive biomarkers of ICI response in breast cancer.

5. Conclusions

To date, PD-L1 assessment in tumor samples remains the only biomarker used to inform immunotherapy decisions in breast cancer. However, as we highlighted here, several technical and biological variables may hamper a univocal interpretation of the employed assays, and the generalization of PD-L1 as an optimal predictive biomarker for ICIs response is far from being established.

Moreover, the use of a single biomarker displays intrinsic limitations and cannot reflect temporal and spatial heterogeneity of both tumor cells and immune microenvironment. In this complex scenario, technological advances in high-throughput sequencing and single-cell spatial analyses offer the opportunity to better understand the biological mechanisms underlying tumor-immune co-evolution and will pave the way for the discovery of a multitude of novel predictive biomarkers.

The integration of these biomarkers with PD-L1 in a “holistic” perspective will be a major step towards precision immune-oncology. Research efforts to develop comprehensive panels of multiple predictive factors, including both tissue- and blood-based biomarkers, may allow clinicians to improve patient stratification with the ultimate aim of allocating individual patients to receive the most appropriate treatments.

Conflict of interest disclosures

LL has served on the advisory boards for: Lilly, Exact Sciences, Italfarmaci, AstraZeneca and Daiichi Sankyo; has received consulting fee from: Exact Sciences and Helsinn; honoraria for speakers’ bureaus from: Gilead, Exact Sciences and EISAI; support for travel, accommodations, expenses from: Lilly and Gilead.

GV has served on the advisory boards for Gilead; has received honoraria for speakers’ bureaus from Novartis, Lilly; support for travel, accommodations, expenses from: Lilly and Pfizer.

GB has received consulting fee from Roche, AstraZeneca, Novartis, MSD, Sanofi, Daiichi Sankyo, and Exact Sciences; honoraria for speakers’ bureaus from Roche, Pfizer, Astra- Zeneca, Lilly, Novartis, Neopharm Israel, MSD, Chugai, Daiichi Sankyo, EISAI, and Exact Sciences; support for travel, accommodations, expenses from Roche, Pfizer, and AstraZeneca; is co-inventor of ‘European patent Application N. 12195182.6 and 12196177.5 titled “PDL-1 expression in anti-HER2 therapy” -Roche- Issued (no compensation provided); has served on the advisory boards for Pfizer, Roche, Daiichi Sankyo, Lilly, MSD, Novartis, AstraZeneca, Genomic Health, EISAI, Gilead, Seagen.

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