Immunotherapy in breast cancer patients:

A focus on the use of the currently available biomarkers in oncology

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Running title: Immunotherapy biomarkers in breast cancer

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ABSTRACT

Immune checkpoint inhibitors (ICIs) have remarkably modified the way solid tumors are managed, including breast cancer. Unfortunately, only a relatively small number of breast cancer patients significantly respond to these treatments. To maximize the immunotherapy benefit in breast cancer, several efforts are currently being put forward for the identification of i) the best therapeutic strategy (i.e. ICI monotherapy or in association with chemotherapy, radiotherapy, or other drugs); ii) the optimal timing for administration (e.g. early/advanced stage of disease; adjuvant/neoadjuvant setting); iii) the most effective and reliable predictive biomarkers of response (e.g. tumor-infiltrating lymphocytes, programmed death-ligand 1, microsatellite instability associated with mismatch repair deficiency, and tumor mutational burden). In this article, we review the impacts and gaps in the characterization of immune-related biomarkers raised by clinical and translational research studies with immunotherapy treatments. Particular emphasis has been put to the documented evidence of significant clinical benefits of ICI in different randomized clinical trials, along with preanalytical and analytical issues in predictive biomarkers pathological assessment.

KEYWORDS: breast cancer, biomarkers, immunotherapy, TILs, PD-L1, mismatch repair, microsatellite instability, tumor mutational burden.

INTRODUCTION

Immune checkpoint inhibitors (ICIs) have broadened the treatment landscape of many solid tumors, including breast cancer [1-3]. Currently approved ICIs are those targeting the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) axis and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) protein [4]. Regrettably, only a minority of breast cancer patients is significantly sensitive to these drugs. In these women, a remarkable number of possible ICI-containing combination strategies has been (and is being) explored, including association with radiotherapy, chemotherapy, anti-angiogenic drugs, targeted therapies, and other ICIs [5, 6]. In this multifaceted scenario, a biomarker-based approach in patients' selection for neoadjuvant or adjuvant immunotherapy is crucial to improve clinical outcomes [7].

The most studied immune-related biomarkers in breast cancer are PD-L1 expression level, distribution of tumor-infiltrating lymphocytes (TILs), high levels of microsatellite instability (MSI) associated with mismatch repair deficiency (dMMR), and degree of tumor mutational burden (TMB) [4, 8]. Despite the largest benefit has been observed in patients with PD-L1^{+} metastatic triple-negative breast cancers (TNBC), ICIs combinations are also being studied in other subtypes and clinical settings. Current efforts focus on developing immunotherapy combinations to convert non-responders to responders, deepening those responses that do occur, and overcoming resistance. Additionally, complementary and/or surrogate biomarkers may have a role in improving the efficacy of ICI-based regimens [9, 10]. Novel means of mutation measurement as comprehensive genomic profiling for identifying alterations across genes are currently under investigation [11].

CTLA-4 upregulation is recognized as an important mediator of tumor immune evasion [12]. Once this antigen is exposed to cytotoxic T-lymphocyte membrane, it competes for and binds to

B7, with a higher affinity than CD28, inhibiting the previously activated pathways. The activation of this pathway leads to the suppression of T cells and functions as a co-inhibitory signal [13]. Of note, CTLA-4 overexpression has been reported in about 50% of breast cancers [14]. Unlike in melanoma and non-small cell lung cancer [15, 16], however, recent clinical studies failed to demonstrate significant benefits of anti-CTLA-4 antibodies in breast cancer therapy both in terms of progression-free Survival (PFS) and overall survival (OS) [17]. The types of antibodies along with the ICIs interaction mechanisms with the specific biomarkers are portrayed in **Figure 1**.

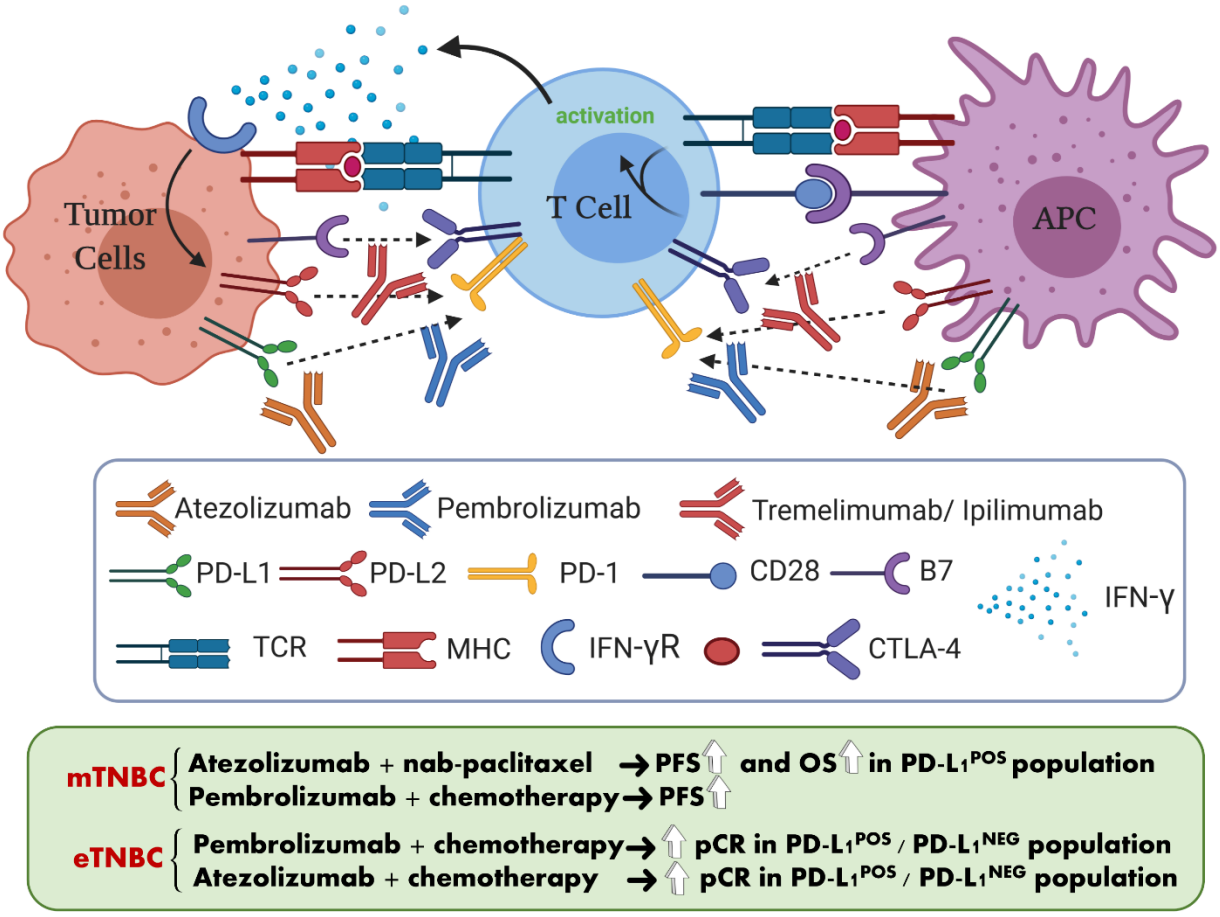

Figure 1. Immune-checkpoint inhibition in breast cancer, biomarkers, therapeutic targets, and clinical applications. Representation of T-lymphocyte activation mechanism caused by tumor-derived antigens. Tumor cells escape immune system activation by overexpressing PD-L1/2 and/or CTLA-4 ligand. ICI antibodies respectively bind to PD-L1, PD-1 and CTLA-4 and block their activation. The clinical setting of Atezolizumab and Pembrolizumab

describe how ICI combination with chemotherapy results in patients' survival and response improvement. ICI, immune-checkpoint inhibitors; mTNBC, metastatic triple-negative breast cancer; eTNBC, early triple-negative breast cancer; TMB, tumor mutational burden; TILs, tumor-infiltrating lymphocytes; PD-1, programmed cell death protein 1; PD-L1/2, programmed death-ligand 1/2; IFNγ, interferon gamma; MHC, major histocompatibility complex; TCR, T-cell receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; OS, overall survival; PFS, progression-free survival; pCR, pathologic complete response.

To date, the naïve vision of a single-feature-based patient selection for immunotherapy has become insufficient [6, 8]. Indeed, in real-life practice the clinical ramifications of breast cancer management are extremely intricate. In this review article, we seek to illustrate the impacts and gaps in the characterization of immune-related biomarkers for immunotherapy prediction in breast cancer. Particular focus has been given to the reliability of currently available tools and possible drawbacks for their pathological characterization.

IMMUNE CHECKPOINT BLOCKADE AND COMBINATION STRATEGIES: STATE OF THE ART

The first approach of immunotherapy in breast cancer has been with adjuvant ICIs monotherapy regimens. It has been widely demonstrated that these drugs may be active, showing objective response rates (ORR) ranging from 2.8% to 21.4%, with higher benefit in TNBC, PD-L1^{+} tumors, and as first-line therapy [18-21]. The KEYNOTE-119 trial (NCT02555657) is the only randomized study that compares the activity of a single ICI agent (i.e. pembrolizumab, an anti-PD-1 monoclonal antibody) versus single agent chemotherapy (i.e. capecitabine, eribulin, gemcitabine, or vinorelbine) for metastatic TNBC (mTNBC). The study failed to meet its primary endpoints (i.e. PFS and OS), but the clinical benefit has been reported for ICI administration in patients showing elevated PD-L1 expression levels (i.e. combined positive score (CPS) ≥20). Of note, 25-30% and 60-70% of breast cancer patients experience secondary/acquired resistance and harbor primary/intrinsic resistance to ICIs, respectively [22, 23]. To overcome these

resistance phenomena, novel combinatorial treatments and alternative therapeutic strategies are currently under investigation. The most important clinical trials investigating the ICI efficacy in breast cancers are summarized in **Table 1**.

NCT Number	Trial Name	PD-L1 Antibody clone	Scoring system	Drug	Phase	Setting	Status	Patients	Breast cancer subtype	Primary outcome	Secondary outcome
NCT02555657	KEYNOTE-119	22C3	CPS	Pembro	III	A	Ac(nr)	622	TNBC	OS	ORR, PFS, DOR, DCR, AEs
NCT02819518	KEYNOTE-355	22C3	CPS	Pembro + CT	III	A, R	Ac(nr)	882	TNBC	AEs, PFS, OS	ORR, DOR, DCR
NCT03036488	KEYNOTE-522	22C3	CPS	Pembro + CT	III	A	Ac(nr)	1,174	TNBC	pCR, EFS	pCR, EFS, OS, AEs,
NCT02447003	KEYNOTE-086	22C3	CPS	Pembro	II	A	C	254	TNBC	ORR, AEs	DOR, DCR, PFS, OS
NCT01848834	KEYNOTE-012	22C3	CPS	Pembro	I	A	C	297	TNBC	AEs, ORR	ORR
NCT02628067	KEYNOTE-158	22C3	CPS	Pembro	II	A	Re	1,595	Any	ORR	DOR, PFS, OS
NCT02425891	IMpassion130	SP142	IC	Atezo + Nab-Pacl	III	A	Ac(nr)	900	TNBC	PFS, OS	CR, PR, DOR, TTD, AEs, ATAs
NCT03125902	IMpassion131	SP142	IC	Atezo + Pacl	III	A	Ac(nr)	600	TNBC	PFS	OS, PA, TTD, ORR, DOR, AEs, C-
NCT03371017	IMpassion132	SP142	IC	Atezo + CT	III	R	Re	572	TNBC	OS	PA, PFS, ORR, DOR, CBR, C-DoR, T
NCT03197935	IMpassion031	SP142	IC	Atezo + CT	III	E	Ac(nr)	324	TNBC	pCR	EFS, DFS, OS, ADAs
NCT02685059	GeparNuevo	SP263	IC	MEDI4736 (Anti PD-L1) + CT	II	E	Un	174	TNBC	pCR	pCR, CR, AEs, OS
NCT02620280	NeoTRIPaPDL1	SP142	IC	Atezo + Carbo + Nab-Pacl	III	E, A	Ac(nr)	278	IDBC	EFS	pCR, COR, DEFS
NCT02129556	PANACEA	22C3	CPS	MK-3475 + Trastu	I/II	A	C	58	$HER2^+$	DLT, ORR	DOR, TTP, DCR, PFS, OS
NCT02924883	KATE2	SP142	IC	Atezo + Trastu emtansine	II	A	C	202	$HER2^+$	PFS, AEs	OS, OR, ATAs
NCT02489448	N/A	SP263	IC	MEDI4736 (Anti PD-L1) + Nab-Pacl + CT	I/II	E	Suspended due to COVID-19	71	TNBC	pCR	Safety and Toxicity
NCT03199885	N/A	N/A	N/A	Atezo + Pacl + Trastu + Pertu	III	A	Re	600	$HER2^+$	PFS	OS, OOR, AEs
NCT03051659	N/A	22C3	MPS	Pembro + EM	II	A	Ac(nr)	88	HR^+	PFS	ORR, OS, IRR, DOR, CBR

Table 1. Ongoing and recently completed clinical trials using immune checkpoint inhibitors (ICI) alone or in combination in breast cancer patients based on PD-L1 status.

Abbreviations: PD-L1, programmed death ligand 1; CPS, combined positive score; IC, immune cell; MPS, modified proportion score; A, advanced/metastatic; R, recurrent; E, early; Ac, active; nr, not recruiting; Re, Recruiting; Un, Unknown; C, completed; Pembro, pembrolizumab; Atezo, Atezolizumab; Pacl, Paclitaxel; CT, chemotherapy; Carbo, Carboplatin; EM, Eribulin Mesylate; Trastu, Trastuzumab; Pertu, Pertuzumab; TNBC, Triple-Negative Breast Cancer; IDBC, Invasive ductal breast carcinoma; HR, Hormone receptor; HER2, Human epidermal growth factor receptor 2; OS, Overall survival; PFS, Progression-free survival; AEs, adverse events; pCR, pathologic complete response; EFS, Event-fee survival; DLT, Dose-limiting toxicity; ORR, objective response rate; DOR, Duration of response; DCR, disease control rate; CR, Complete response; PR, Partial response; TTD, Time to deterioration; ATAs, Anti-therapeutic antibodies; PA, Participants alive; C-DoR, Duration of confirmed response; CBR, Clinical benefit rate; ADAs, Anti-drug antibodies; COR, Clinical objective response; DEFS, Distant event free survival; IRR, Immune response rate; OOR, Overall objective response; N/A, Not available. Information has been obtained from clinicaltrials.gov.

Anti-PD-L1 immunotherapy in advanced/metastatic settings

To date, the only immunotherapy regimen approved in breast cancer patients is the combination of the anti-PD-L1 monoclonal antibody atezolizumab and nanoparticle albumin-bound (nab)-paclitaxel as first-line treatment for $PD\text{-}L1^+$ mTNBC [24]. This approval followed the results of the IMpassion130 (NCT02425891), a phase III randomized trial that enrolled patients with treatment-naïve mTNBC to receive either atezolizumab plus nab-paclitaxel or nab-paclitaxel and

placebo [24]. OS and PFS were co-primary endpoints of the study. The trial had a hierarchical statistical design, requiring benefit in the intent-to-treat (ITT) population to formally test the effect in subgroups by PD-L1 status. In this study, PD-L1 was assessed by immunohistochemistry (IHC) using the VENTANA PD-L1 (SP142) Assay (Roche). The positivity was defined when PD-L1 was expressed in ≥1% of immune cells (IC) [24]. This study showed that the addition of atezolizumab to nab-paclitaxel significantly improves PFS in both the ITT population and the PD-L1$^+$ population. The OS analysis in the ITT population was negative but suggested a clinically meaningful OS benefit with atezolizumab plus nab-paclitaxel in patients with PD-L1 IC+ disease, leading to a 7-month OS improvement [24, 25]. These results led the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to approve the combination of atezolizumab plus nab-paclitaxel in this setting. The IMpassion131 trial (NCT03125902) investigated the combination of atezolizumab and paclitaxel vs. paclitaxel alone as first-line therapy for patients with mTNBC. The results of this study have been recently presented and showed neither a PFS nor OS advantage when atezolizumab was added in both PD-L1$^+$ and ITT populations. The ongoing randomized Phase III IMpassion132 trial (NCT03371017) is evaluating the efficacy of first-line atezolizumab in combination with chemotherapy vs. chemotherapy alone for inoperable locally advanced/mTNBC relapsing within 1 year after standard (neo)adjuvant anthracycline and taxane chemotherapy [26, 27].

Anti-PD-1 immunotherapy in advanced/metastatic settings

The KEYNOTE-355 study has recently shown a benefit with the combination of first-line pembrolizumab and chemotherapy in previously untreated mTNBC (NCT02819518) [28]. In this phase III trial, patients were randomized to receive chemotherapy (i.e. taxane or carboplatin/gemcitabine) with placebo or chemotherapy plus pembrolizumab. In this study, PD-L1 was assessed by using the PD-L1 IHC 22C3 pharmDx assay (Agilent) [29]. Patients were stratified for PD-L1 expression (CPS ≥1 vs CPS <1). The combination of chemotherapy and

pembrolizumab significantly improved PFS compared with chemotherapy alone in patients with CPS ≥10, whereas patients with a CPS <10 did not seem to benefit from the addition of pembrolizumab to chemotherapy. These findings suggest a role for the addition of pembrolizumab to standard chemotherapy for the first-line treatment of mTNBC [28]. The estimated study completion date of the KEYNOTE-355 study is January 2022.

Immune-checkpoint inhibition in early stage

Metastatic breast cancer is commonly not inflamed due to the immune escape mechanisms occurring during the disease progression [30-33]. Hence, an important point is whether ICIs should be given as early as possible, thus including neoadjuvant therapy, in the course of the disease. Two recently presented trials combining ICIs with chemotherapy in early-stage TNBC have shown promising results. The KEYNOTE-522 (NCT03036488) trial randomized patients with TNBC suitable to neoadjuvant treatment to receive chemotherapy with carboplatin/paclitaxel followed by anthracycline/cyclophosphamide with or without pembrolizumab as neoadjuvant, followed by surgery, plus nine more cycles of pembrolizumab or placebo in the adjuvant setting [34]. In this trial, the addition of pembrolizumab led to a statistically significant increase in pathologic complete response (pCR) rate, both in ITT population (51.2% vs 64.8%, p=0.00055) and PD-L1^{+} population (54.9% vs 68.9%). Interestingly, pembrolizumab improved pCR rate also in patients with PD-L1^{-} tumors (30.3% vs 45.3%) [34]. This study has also shown a positive trend in terms of event-free survival (EFS), although data are not mature yet (hazard ratio (HR), 0.63; 95% CI, 0.43–0.93). An important neoadjuvant trial is the IMpassion031, which randomized patients with TNBC to receive chemotherapy with nab-paclitaxel followed by anthracycline/cyclophosphamide with atezolizumab or placebo as neoadjuvant, followed by surgery, plus 11 more cycles of atezolizumab or placebo continued in the adjuvant setting. The co-primary endpoint of the study, pCR in the ITT population, is significantly in favor of adding atezolizumab to

chemotherapy (41.1% vs 57.6%, P = 0.0044). The other co-primary endpoint, pCR in the PD-L1^{+} population, is also significant in favor of adding atezolizumab (49.3% vs 68.8%). Here again, the addition of atezolizumab increased the pCR rate also in the PD-L1^{-} population (34.4% vs 47.7%). At subgroup analyses, both in KEYNOTE-522 and IMpassion031 trials, patients with node-negative disease seem to derive less benefit with such combination.

Studies with negative results

Despite the above-mentioned significant results, research of ICIs in breast cancer is paved of negative studies. The GeparNuevo trial (NCT02685059), a randomized phase II study investigating durvalumab in addition to an anthracycline taxane-based neoadjuvant therapy in early TNBC, failed to demonstrate an increased pCR rate in the overall population (A randomized phase II study investigating durvalumab in addition to an anthracycline taxane-based neoadjuvant therapy in early triple-negative breast cancer: clinical results and biomarker analysis of GeparNuevo study) [35]. Improved pCR rate was highlighted in the subgroup treated with durvalumab started two weeks before chemotherapy. However, this analysis was underpowered for significance testing. The NeoTRIPaPDL1 trial (NCT02620280) explored the addition of atezolizumab to neoadjuvant carboplatin/nab-paclitaxel followed by surgery and then adjuvant anthracycline/cyclophosphamide [36]. This study did not report significant differences in pCR rates among patients who did or did not receive atezolizumab (43.5% vs 40.8%; P=.66). However, it is worth reminding that pCR was a secondary endpoint of the study, while EFS data, which was the primary endpoint, is still awaited [36].

Immunotherapy in other breast cancer subtypes

Although ICIs administration has been predominantly studied in TNBC, some studies have been carried out also in other metastatic breast cancer subtypes. In hormone receptor (HR)$^{+}$/HER2^{-} breast cancer patients, the efficacy of ICIs as monotherapy seems to be very limited [37, 38].

Recently, a multicenter randomized phase II trial evaluated the addition of pembrolizumab to eribulin vs. eribulin alone [39]. In this study, patients with previously treated HR^+/HER2- disease did not benefit from the addition of ICI to chemotherapy. Combinations of ICIs with other agents (e.g. CDK4/6 inhibitors) have also been explored with limited results [40, 41]. In the $HER2^+$ subtype, the combination of an ICI plus an anti-HER2 agent has been firstly assessed in the PANACEA trial [42]. This study included patients with metastatic $HER2^+$ breast cancer who progressed after trastuzumab-based therapy. These patients, regardless of the PD-L1 status, received a combination of pembrolizumab and trastuzumab. An ORR of 15% was highlighted among patients with PD-$L1^+$ tumors, whereas no responses were observed among patients with PD-$L1^+$ tumors. More recently, the KATE2 study evaluated whether the addition of atezolizumab to trastuzumab emtansine (T-DM1) improved efficacy in patients progressed on previous therapy with trastuzumab and a taxane [43]. The study failed to demonstrate a PFS benefit in the ITT population [43]. However, a trend towards improved OS has been observed in patients with PD-$L1^+$ tumors treated in the atezolizumab arm [43]. Currently, a multicenter randomized phase III trial is going to test the efficacy of first-line trastuzumab, pertuzumab, and paclitaxel with or without atezolizumab (NCT03199885) in the setting of $HER2^+$ breast cancers.

Small-molecule immune checkpoint inhibitors

Albeit the majority of the ICIs are monoclonal antibodies, small molecule immuno-oncology inhibitors are actively being pursued by pharmaceutical companies and research groups [44-46]. Most of these low-molecular-weight compounds have been designed for inhibiting the PD-1/PD-L1 interaction overcoming several shortcomings of monoclonal antibodies (e.g. long half-life, adverse effects, low permeability, costs of production and treatment) [47, 48]. Recent studies implemented biochemical and cell-based bioassays to determine the binding, activity, and cytotoxicity of different inhibitors such as BMS-1001, BMS-1166, MS-103, BMS-142 and Aurigene-1 [49, 50]. Most of these compounds are capable of binding specifically to PD-L1,

while they show increased inhibitory activity of the PD-1/PD-L1 interaction and low toxicity levels [49, 50]. Additionally, Konieczny *et al.* reported the development of a novel compound that is more potent and smaller in size compared to others reported in the literature, with improved solubility and nontoxic activity to cells even at high concentrations [51]. CA-170 is an orally available small molecule that directly targets the PD-L1/PD-L2 and V-domain Ig suppressor of T cell activation (VISTA) immune checkpoints and results in activation of T cell proliferation and cytokine production [52]. The safety and pharmacokinetic profile of this small molecule has been tested in a recently completed phase I clinical study involving patients with advanced malignant tumors including TNBC, mesothelioma, melanoma, non-small cell lung cancer, renal cell carcinoma, Hodgkin lymphoma, head and neck cancer, colorectal cancer, gastric cancer, bladder cancer, and ovarian cancer (NCT02812875) [53]. Lessons learned from the development of these agents can accelerate the development of next-generation inhibitors to optimize the therapeutic index, overcome drug resistance, and establish combination therapies. However, further translational and clinical studies are warranted to define whether women with breast cancer would benefit of these drugs.

CLINICOPATHOLOGIC FEATURES RELATED TO IMMUNOTHERAPY RESPONSE

Among breast cancer subtypes, TNBC is usually characterized by higher TILs, PD-L1 expression, and high TMB, suggesting a potential role for immunotherapy [54]. However, TNBCs are extremely heterogeneous and encompass a multitude of tumor types with different morphology, molecular features, immunogenicity, and responses to therapies [55-57]. Surrogate and/or complementary biomarkers, coupled with tumor-specific guidelines and testing methods, might potentially improve the extremely diffucult task of breast cancer patients selection for immunotherapy. The available immune-related biomarkers for breast cancers, and their current prognostic and predictive applications, are shown in **Figure 2**.

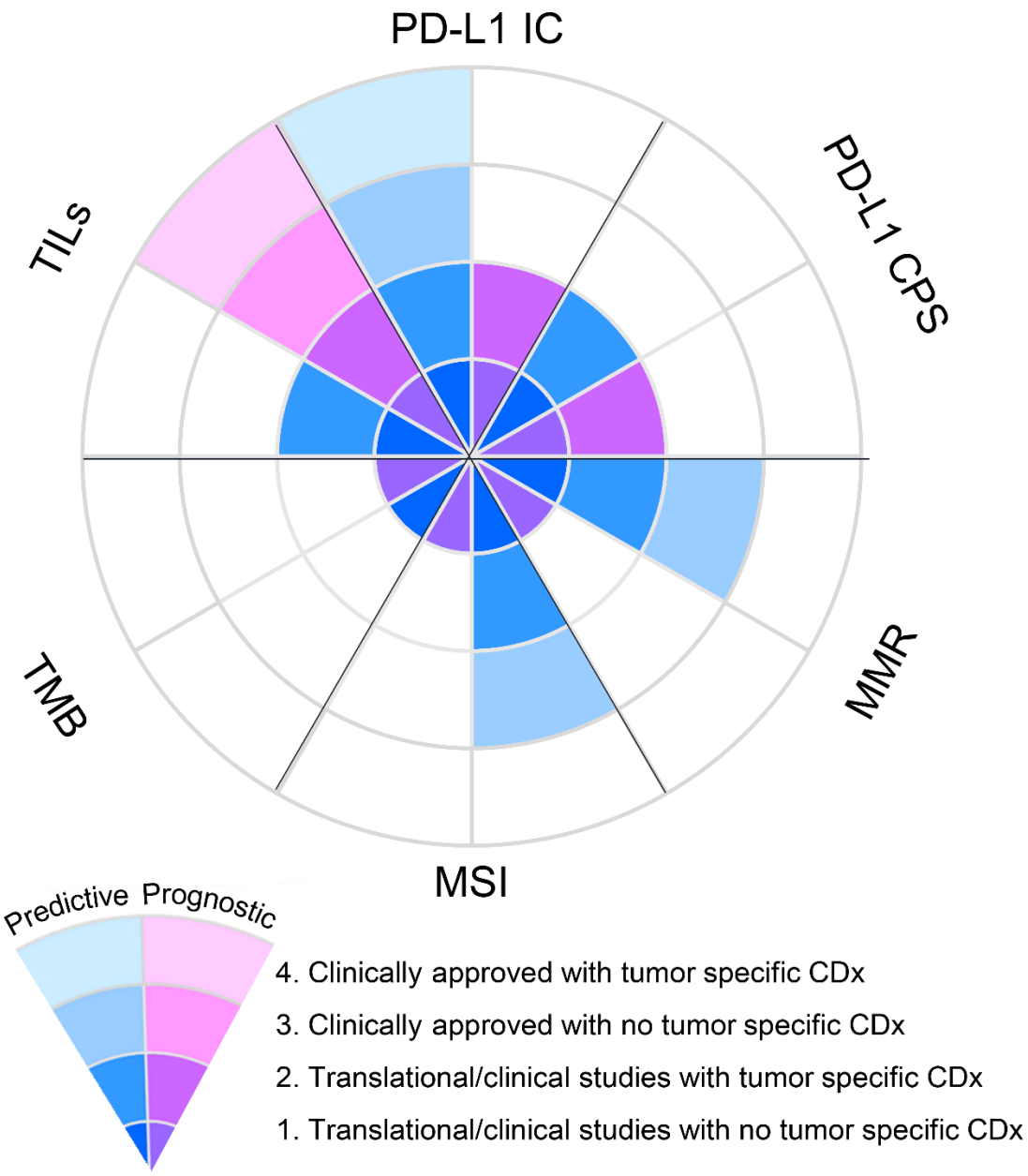

Figure 2. Schematic representation of the main prognostic and predictive biomarkers along with their clinical significance in breast cancer immuno-oncology. Each circle represents the possible application of the various biomarkers in clinical practice, as depicted in the legend. CDx, companion diagnostic test; PD-L1, programmed cell death ligand 1; IC, immune cell; CPS, combined positive score; MMR, mismatch repair; TMB, tumor mutational burden; MSI, microsatellite instability; TILs, tumor-infiltrating lymphocytes.

Tumor-infiltrating lymphocytes (TILs)

TILs are mononuclear immune cells discovered within tumor tissue in most types of solid tumors, including breast cancer. They are mainly composed of cytotoxic $CD8^+$ T cells, helper $CD4^+$ T cells, and natural killer (NK) cells [58]. Cancer-specific mutations and/or neoantigens released in the microenvironment by damaged cancer cells may activate the TILs anti-tumor immune response [59-62]. Most of the studies assessed TILs on a single hematoxylin and eosin (H&E)-stained tumor section, dividing the stromal and intratumoral compartments. Intratumoral TILs (iTILs) directly infiltrate tumor cell nests, whereas stromal TILs (sTILs) are found within the stromal tissue of the tumor. The latter are considered a more reliable biomarker in predicting response to therapy and patient outcome [63-65]. Both compartments may comprise a combination of different lymphocyte subtypes, with a predominant T-cell population. International cooperative efforts are ongoing to maximize reproducibility of this putative prognostic and predictive biomarker [63].

TILs have an established prognostic role in TNBC and $HER2^+$ early/locally advanced breast cancer patients. In these subtypes, a correlation between an increased number of sTILs and outcomes (disease-free survival (DFS) and OS) was demonstrated in multiple retrospective-prospective studies [66-71]. Data on the correlation between TILs and response to immunotherapy were investigated in a few recent clinical trials. In the mTNBC, higher TILs concentrations were associated with improved response in patients treated with pembrolizumab monotherapy in the phase II KEYNOTE-086 study [72, 73]. High TILs levels were also associated with significant improvement in ORR (Odds ratio OR) 1.26, 95% CI 1.03- 1.55, p=0.01) and disease control rate (DCR) (OR 1.22, 95% CI 1.02-1.46, p=0.01). In the PANACEA phase Ib/II trial, 58 metastatic $HER2^+$ breast cancer patients received a combination of trastuzumab and pembrolizumab; a significantly greater lymphocytic infiltration was observed in responders and patients with longer disease control [74]. In a systematic review and meta-

analysis investigating predictive factors of ICIs efficacy in metastatic breast cancer, 27 studies with 1746 patients were included [75]. Despite only a few trials reported TILs evaluation, TILs ≥ 5% (OR = 2.53, p = 0.002) and high infiltrated $CD8^+$ T-cell level (OR = 4.33, p = 0.006) emerged as predictors of immune checkpoint therapy response, in terms of ORR. Of note, in a phase I/II trial (NCT02489448) the safety and efficacy of concurrent durvalumab with weekly nab-paclitaxel followed by dose-dense doxorubicin and cyclophosphamide as neoadjuvant therapy for stage I-III TNBC were investigated [76]. TILs counts were available for 52 out of 57 patients; no significant differences in response to therapy were observed according to TILs value. This may be partly due to the complexity of the immune microenvironment, which is shaped by competing for co-stimulatory and co-inhibitory signals [77]. Taken together, despite their significant prognostic role, TILs role as an independent predictive factor for immunotherapy is still not ready for prime time.

Programmed death-ligand 1 (PD-L1)

The interplay between PD-1 and its agonist PD-L1 could serve as an escape mechanism for tumors to evade antigen-specific T-cell immunologic responses [78, 79]. In breast cancer, PD-L1 expression is often associated with poor clinicopathologic features, high TILs count, and triple-negative phenotype (**Figure 3**) [80, 81].

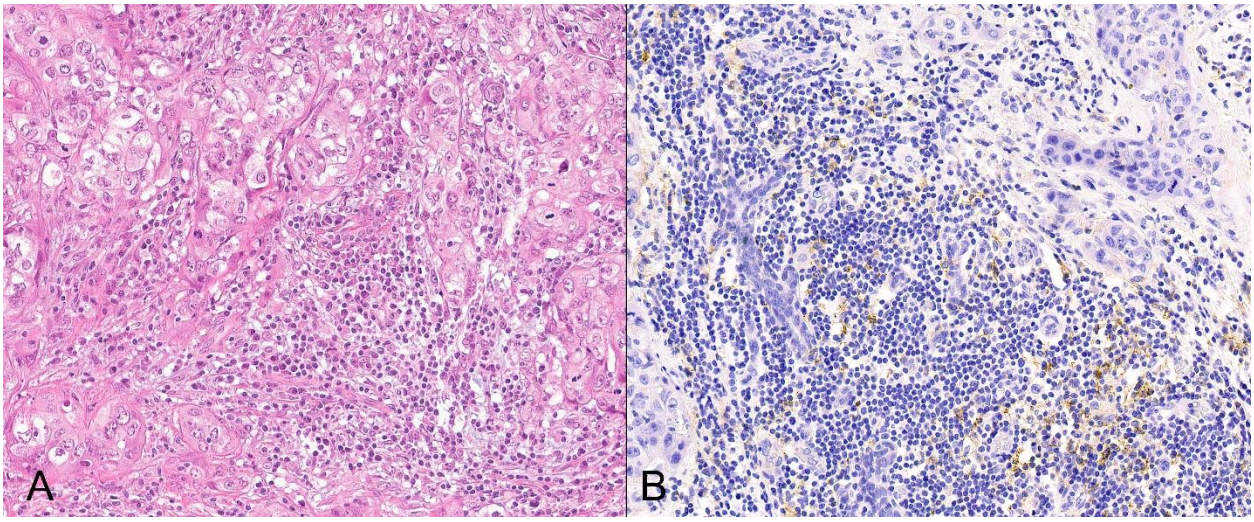

Figure 3. Representative micrographs showing a case of poorly differentiated infiltrating triple-negative breast cancer of no special histological type. This tumor showed a prominent stromal infiltrate of immune cells, being classified as TILs-high (A). Given that both tumor cells and TILs showed PD-L1 immunoreactivity, albeit with different degrees of staining intensity (i.e. faint in tumor cells, mild-moderate in TILs), the combined positive score (CPS) was scored >20 (B). Original magnification, 200×.

The PD-1/PD-L1 axis is considered a promising immunotherapeutic target in several solid tumors [82]. PD-1/PD-L1 blockage leads to the activation of T cells that can recognize and attack cancer cells [83, 84]. So far, PD-L1$^+$ and treatment-naïve TNBC patients have been the most suitable candidates for ICI therapy in breast cancer [85]. Preliminary evidence regarding the clinical activity of pembrolizumab was firstly reported in the KEYNOTE-012 trial (NCT01848834). This study included previously treated, advanced TNBC patients showing an ORR of 18.5%, and a median time to response of 17.9 weeks (range, 7.3 to 32.4 weeks) [18]. Subsequently, the phase II KEYNOTE-086 (NCT02447003) examined the efficacy and safety of pembrolizumab monotherapy in two cohorts of patients. The first cohort included patients exposed to one or more prior lines of systemic treatment for metastatic disease regardless of PD-L1 expression, while the other cohort involved PD-L1$^+$ patients with non-systemic anticancer therapy [20, 73]. The PD-L1$^+$ cohort showed an ORR of 23% (95% CI 14–36). This suggested a promising antitumor activity of pembrolizumab as first-line therapy for PD-L1$^+$, mTNBC [5, 20, 73]. Antibodies for PD-L1 assessment by IHC have different interpretation guidelines, which lead to the identification of different patient populations [86]. A *post hoc* analysis of IMpassion130 assessed the analytical concordance of PD-L1 evaluation by SP142, SP263 (VENTANA), and PD-L1 22C3 (Dako) IHC assays [87]. PD-L1 positivity was considered as, IC ≥ 1% for SP142 and SP263 assays, and CPS ≥ 1 for the 22C3 assay. These findings showed that the 22C3 and SP263 assays identified more patients with PD-L1$^+$ tumors (81% and 75% respectively) compared to SP-142 (46%) [87]. However, the absolute benefit for the addition of atezolizumab for the samples that were only positive with 22C3 assay (PFS: 1.7 months) or SP-

263 (PFS: 1.6 months) was considered different in comparison with those that were only positive for SP-142 (PFS: 4.2 months) [86]. Accordingly, the SP-142 antibody showed the greatest clinical benefit in the TNBC, PD-L1^{+} patients.

Mismatch repair and microsatellite instability

The MMR system recognizes and corrects ribonucleotide misincorporation errors generated during DNA replication and recombination, preserving the overall replication fidelity and genome stability [88, 89]. MMR deficiency (dMMR) may be generated due to mutations and/or epigenetic silencing of MMR genes, potentially leading to MSI, a phenotype characterized by alterations in the length of microsatellite regions [90, 91]. It has been observed that dMMR tumors harbor several mutations in cancer-related genes resulting in high neoantigen formation and subsequent T-cell recruitment, making these patients likely sensitive to ICIs [92, 93]. Relying on this rationale, the FDA granted the tissue-agnostic approval of pembrolizumab in dMMR or MSI-high solid tumors [94]. Although this approval is reasonable across different types of cancer where these biomarkers are well-validated [95, 96], in breast cancer, further investigation of the MMR and MSI status is warranted. MMR deficiency shows a relatively low frequency in breast cancer, being present in approximately 2% of cases. However, this is a controversial matter due to the lack of companion diagnostic (CDx) tests and/or tumor-specific guidelines for MMR analysis [9, 97-101]. Most of the protocols that have been employed to date are locally developed using different methods such as the sequencing of microsatellite markers, next-generation sequencing (NGS), and IHC for the four MMR proteins. A recent study demonstrated that dMMR can be used as a prognostic and likely predictive biomarker, but without being interchangeable with MSI as in other tumor types [98]. Additionally, a case study of a woman with metastatic, ER^{+}, HER2^{-} breast cancer, showed durable complete remission after treatment with pembrolizumab [102]. Although dMMR cases are infrequent in breast cancer, there is an urgent need to identify and select even that fairly low proportion of patients who could benefit

from immunotherapy. To accomplish this goal a standardization of MMR analysis with tumor-specific guidelines would be required. In this respect, the phosphatase and tensin homolog (PTEN) was recently proposed as a complementary biomarker for MMR testing in breast cancer [9]. Indeed, this gene is not only involved in cell growth, proliferation, and survival, but it is also implicated in the modulation of the DNA damage response and in tumor immune microenvironment modeling [10]. Hence, PTEN expression analysis can be employed to identify MMR-proficient breast cancers, thus overcoming the lack of overlapping between MMR IHC and MSI analyses [9]. Additional clinical trials and prospective translational research studies are needed to provide reliable results for MMR testing in the selection of breast cancer patients for immunotherapy.

Tumor mutational burden

The term TMB refers to the absolute number of somatic mutations per mega-base (mut/Mb) arising in tumor-coding regions [103]. Given that patients with a high-TMB (hTMB) are more prone to achieve clinical benefit from ICI treatment, this biomarker has been proposed in predictive settings [104, 105]. Despite not all mutations result in immunogenic neoantigens, the higher is TMB the greater is the probability of tumor neoantigen production and, therefore, the immune triggering [106]. Breast cancers display intermediate levels of mutational load [107], with a significantly higher median TMB in TNBC compared to other receptor subtypes (TNBC> $HER2^+$ >ER^+/ $HER2^-$) [108]. Although debated, high TMB value (>10mut/Mb) are observed in less than 5% of breast cancers, with prevalence varying according to breast cancer subtype [108, 109].

The correlation between TMB levels and the expression of immune-related biomarkers is still controversial. In pooled analyses of different cancer types as well as in breast cancer, a positive association of TMB and TILs was observed [59, 109]. Additional studies failed to detect this

correlation in TNBC [110, 111]. Furthermore, unlike in other solid tumors [112], no significant association between PD-L1 positivity and hTMB was observed in breast cancer [113, 114]. Interestingly, a hTMB status is recurrent in breast cancers with DNA repair deficiency due to alterations in the MMR pathway. Mutations in replicative DNA polymerases (e.g. *POLE* and *POLD1*), and deficit in the homologous recombination repair system occurrence are also associated to hTMB [108, 109, 115, 116]. Moreover, an association between misregulation of DNA-mutating enzymes activity (i.e. APOBECs) and a hypermutator phenotype has been identified in breast cancer [108, 117]. There is recent evidence that TMB levels may double in the metastasis compared to the primary tumor in breast cancer patients, maybe due to treatment-associated selective pressure [108, 110, 114, 117]. Moreover, hTMB level is usually observed in older breast cancer patients [110, 114]. Among patients with mTNBC treated with ICI, hTMB was significantly associated with longer survival independently of PD-L1 status, while TMB predicted pCR after neoadjuvant ICI in early TNBC [110, 118]. Remarkably, TMB levels detected in circulating tumor DNA seem to mirror those observed at the tissue level, making TMB testing on liquid biopsy a promising and less invasive strategy [119].

Despite the potential clinical application, TMB role in breast cancer immuno-oncology still requires further studies. Hence, different pre-analytical and analytical variables can dramatically affect its measurement and reproducibility. Several studies suggested that TMB assessment using targeted NGS panels, instead of whole exome sequencing (WES), is more reliable [120]. Regrettably, the heterogeneity of the possible targeted panels may affect TMB counts. Another crucial question regards the definition of a cutoff value to identify hTMB and therefore patients that could benefit from immunotherapy. TMB is a continuous variable and placing a threshold may be challenging. Different cutoffs values (as ≥10, 16, 20 mut/Mb detected by F1CDx panel) have been proposed by several groups to reach the best accuracy in identifying ICI responsive patients [121-123]. In June 2020 the FDA approved a cutoff of ≥10 mut/Mb, measured by

F1CDx, for labeling hTMB in solid tumors, predictive of pembrolizumab response, based on the KEYNOTE-158 clinical trial [124]. Regrettably, no additional guidelines using other TMB tests and/or different ICI protocols are currently available [125]. Notably, the European Society for Medical Oncology (ESMO) has recently recommended TMB testing in several tumors to predict pembrolizumab response but not yet in breast cancer [126].

WHAT SAMPLE TO TEST AND WHICH ASSAY TO USE: A STILL UNADDRESSED DILEMMA

The evaluation of PD-L1 expression using IHC on tumor tissue sections is the most studied biomarker for immunotherapy in different tumor types [4, 62, 127, 128]. In this respect, a variety of antibody clones and scoring systems is currently available. Similar analytical performance has been reported for SP263, 22C3 and 28-8 antibodies considering tumor cells expression; while a lower number of cells are stained using SP142 assay [129, 130]. However, variability was observed across the four assays evaluating immune cell expression [129]. In the IMpassion130 trial, PD-L1 expression using the SP142 antibody clone predicted the benefit from the combination of atezolizumab and nab-paclitaxel [131]. In a retrospective analysis of the trial population, a higher number of PD-L1$^+$ tumors have been identified using Dako 22C3, and SP263 as compared to SP142 assay. However, small absolute benefit and no median OS differences were seen among patients with PD- L1$^+$ tumors as identified only by 22C3 or SP263 antibodies [86, 87]. VENTANA PD-L1 (SP142) IHC assay was the only approved companion diagnostic test for atezolizumab using the tumor-infiltrating IC score with a cut-off of 1% [132]. IC score is defined as the proportion of tumor area occupied by PD- L1$^+$ immune cells located in the intratumoral and contiguous peritumoral stroma; tumors are considered PD-L1$^+$ showing ≥1% IC score [133-135].

Laboratory-developed or commercial microsatellite markers PCR-based tests are used for the identification of MSI. The analysis of MMR proteins expression (i.e. MLH1, PMS2, MSH2 and MSH6) using IHC has been performed to ascertain dMMR tumors [88, 97]. Moreover, NGS-based approaches have been increasingly adopted to assess MSI in the context of comprehensive genomic profiling [136]. Most methods have been largely validated for specific tumor types (i.e. colorectal cancers), demonstrating good concordance between MSI and MMR status [137]. Indeed, for patients with sporadic cancers that fall in the Lynch syndrome-associated tumor spectrum, IHC MMR protein evaluation is recommended as the first-choice method followed by MSI PCR-based testing for cases with equivocal or indeterminate results [138]. However, no companion diagnostic test and/or tumor-specific guidelines are currently available for MSI/MMR testing and few data have been reported about the performance of these different methods in breast cancers [98, 136].

Given the immunological heterogeneity between primary and metastatic cancer and the dynamic nature of tumor-immune interplay [33, 128], samples from metastatic/relapse breast cancer may be required for the assessment of immunotherapy biomarkers. In the metastatic setting, a biopsy of an accessible metastatic lesion is currently recommended for patients with advanced breast cancers, although samples from primary tumors are considered acceptable for the analysis of PD-L1 and/or MSI/MMR [134, 135]. Core biopsy tissue specimens should be managed according to standard laboratory procedures to have sufficient material for diagnostic purposes, to reassess biological markers and, when indicated, to test other predictive factors. Although cytology and bone metastasis samples are adequate for DNA extraction and molecular profiling, they are currently considered suboptimal for PD-L1 analysis [135, 139]. The IC score with VENTANA PD-L1 (SP142) Assay should be performed on a tissue sample with at least 50 viable tumor cells [135]. Pre-analytical factors that may affect both the quality of DNA and immunostaining include the time of tissue fixation, type of fixative, and storage condition of

the tissue blocks [135, 140]. In some tumors, liquid biopsy has been shown as a valid alternative for biomarkers analysis especially when tissue specimens are insufficient, or tissue biopsy is not feasible/available [141]. TMB and MSI can also be assessed on cfDNA using broad NGS panels [142, 143]. Moreover, analysis of PD-L1 expression on circulating tumor cells and PD-L1 quantification in plasma sample have been reported, with promising results [144]. However, the implementation and the role of these tests in the clinical setting still require further investigation.

FINAL REMARKS

Immunotherapy has changed the way we treat a subset of patients diagnosed with advanced TNBC. The combination of atezolizumab and nab-paclitaxel is currently the standard first-line therapy in patients with metastatic TNBC who have a PD-L1-positive disease. Although the current approval is limited to a minority of patients, other treatment modalities are showing promising results and might become concrete options in the near future, thus expanding the potential applicability of immunotherapy in breast cancer. Several clinical trials are testing in mTNBC combinations of PD-1/PD-L1 inhibitors and different agents including radiation therapy, targeted agents, and other immunotherapy agents. Furthermore, there is available evidence suggesting that radiation therapy may trigger an immune response through interferon signaling, thus causing immunogenic cell death [145-147]. The activation of dendritic cell and their interaction with T cells may induce the so-called abscopal effect, which is represented by the regression of metastases distant from the irradiated site [147]. Among targeted agents that may be combined with PD-1/PD-L1 inhibitors, particular interest relies on PARP inhibitors, as they have been recently approved for patients with BRCA1/BRCA2 germline mutations. Such combination is also based on a solid scientific rationale that shows how BRCA-mutated tumors are more immunogenic [148-150]. PARP inhibitors trigger interferon signaling and antitumor

immunity response, through the activation of the STING pathway, thus leading to CD8+ T cells infiltration into the tumor microenvironment [150]. The combination of PARP inhibitors and ICIs seems to play a synergistic activity, as seen in clinics. [151, 152] Moreover, immunotherapy agents other than PD-1 or PD-L1 inhibitors have demonstrated promising synergistic activity in breast cancer. As an example, we may cite the lymphocyte activation gene-3 (LAG-3), which acts synergistically with PD-1 to ensure immune homeostasis, through modulation of cytokine secretion and subsequent lymphocyte activation inhibition [153]. Durable responses have been documented in patients with advanced TNBC, who have been treated – whiten a phase I trial - with a combination of spartalizumab (a monoclonal antibody blocking interaction of PD-1 with PD-L1 and PD-L2) and LAG525, a monoclonal antibody blocking binding of LAG-3 to major histocompatibility complex class II [154]. More recently, we have also witnessed the benefit – in terms of increased pCR rate - provided by the addition of PD-1/PD-L1 inhibitors to standard treatment in the neoadjuvant setting; however, survival data are still immature [155].

These data, combined with the evidence that immune escape progressively augments while the disease advances, may lead us to think whether anticipating immunotherapy as earlier as possible in the course of the disease would be more beneficial [30-33, 156]. On the other hand, when we talk about immunotherapy, especially in the curative setting, we cannot forget the potential immune-related adverse events, which require an integrated approach for their prevention and management. The way to optimize immunotherapy benefits in breast cancer is still long. Correlative studies in patients treated with immunotherapy are needed to select the best strategies for further development as well as to identify biomarkers able to expand the group of patients more likely to benefit.

Consent for Publication

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Conflict of Interest

C.C. has received honoraria for consulting/advisory role/speaker bureau from Novartis, Eli-Lilly, Pfizer, Roche. E.G.R. has received honoraria for advisory role/speaker bureau from Thermo Fisher Scientific, Roche, Novartis, AstraZeneca. M.I. has received consultation honoraria from Errekappa Euroterapici S.p.a. U.M. reports personal fees (for service in the speaker bureau and as an advisor) from Boehringer Ingelheim, AstraZeneca, Roche, MSD, Amgen, Merck, Eli Lilly, Thermofisher, Diaceutics. N.F. has received honoraria for consulting/advisory role/ speaker bureau from Novartis, Merck Sharp & Dohme (MSD), and Boehringer Ingelheim. These companies had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, and/or in the decision to publish the results. All the other authors declare no conflicts of interest.

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