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Biomarkers of response and resistance to immune checkpoint inhibitors in breast cancer

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ABSTRACT

Immune checkpoint inhibitors (ICIs) have recently been approved in subsets of patients with breast cancer (BC). Currently, programmed death ligand 1 (PD-L1) immunohistochemistry is used as a biomarker of response for metastatic triple negative breast cancer (TNBC). Other tumor-agnostic indications in metastatic BC include high tumor mutational burden and mismatch repair deficiency. In early TNBC, the ICI pembrolizumab is routinely added to neoadjuvant chemotherapy, yet no biomarker is currently available to predict response or resistance. Further, while luminal BC is often thought to be immune-depleted, preliminary efficacy data in early-stage disease suggests that the addition of ICIs to neoadjuvant chemotherapy can significantly improve rates of pathological complete response. However, not all patients will benefit from ICI treatment and it also comes with significant treatment toxicities. This review will describe biomarkers of response and resistance to ICIs in BC. These currently include tumor infiltrating lymphocytes, homologous recombination deficiency, CD274 gain or amplification, estrogen receptor and/or progesterone receptor expression, more precise tumoral immune characterization, gene expression analysis, and the T-cell receptor repertoire. Although still investigational, these approaches hold the potential to advance personalized medicine by tailoring the use of ICIs to BC patients who will benefit.

1. Introduction

Immune checkpoint inhibitors (ICIs) have revolutionized the treatment landscape of numerous cancer types. They stimulate the anti-tumor response by inhibiting immune checkpoints that are important for tolerance, such as programmed cell death 1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) [1,2]. Initially, breast cancer (BC) was considered less responsive to ICIs due to its lower immune infiltration. However, substantial variability exists among subtypes of BC, with triple negative breast cancer (TNBC) exhibiting the highest neoantigen load and immune infiltration followed by human epidermal receptor 2 (HER2)--positive BC and luminal BC [3,4]. Following positive phase III clinical trials, ICIs in combination with chemotherapy have become the standard of care for early and PD-L1 positive metastatic TNBC [5–7]. Additional indications are high tumor mutational burden (TMB) and deficient mismatch repair (dMMR), all with tumor agnostic approval [8].

The goal of identifying biomarkers of response and resistance to ICIs is threefold. Firstly, ICIs have significant treatment toxicities and financial costs and their use must be tailored to patients who will truly benefit [9,10]. Secondly, there is a strong rationale for using ICIs beyond TNBC in a subset of luminal and HER2+ BC and identifying reliable

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biomarkers could enable a greater proportion of patients to benefit [11,12]. Finally, the discovery of a potential new druggable target could enhance ICI efficacy and numerous modalities are currently being investigated [13]. This article will start by reviewing the relevant trials in BC that described PD-L1 immunohistochemistry (IHC) and tumor-infiltrating lymphocytes (TILs) as biomarkers of response to ICI, followed by other investigational approaches to identifying additional biomarkers of response and resistance to ICIs.

2. PD-L1 IHC and TILs

The presence of TILs in the tumor microenvironment (TME) reflects an ongoing anti-tumoral host immune response and is therefore utilized as a predictive and prognostic biomarker in both early and advanced BC [4]. TILs are categorized as either stromal (sTILs) or intratumoral (iTILs), with sTIL evaluation demonstrated to be more easily reproducible between studies as it is calculated as the percentage of lymphocytes and plasma cells in the intratumoral stromal area compared to the total stromal area, based on analysis of a single hemoxylin and eosin stained tumor section [14,15]. Higher TILs are observed in early TNBC and HER2+ BC compared to luminal BC, and in the localized setting are associated with improved outcomes in the former [15,16].

PD-1 is a transmembrane protein located on the surface of many cell types, and its ligand PD-L1 is situated on certain immune cells. The interaction between PD-1 on activated T-cells and PD-L1 leads to immunosuppression, a mechanism to prevent autoimmunity [17,18]. This immune checkpoint is exploited by tumor cells to escape immune targeting. Monoclonal antibodies against PD-1 (e.g. pembrolizumab, nivolumab, dostarlimab) and PD-L1 (e.g. atezolizumab, avelumab, durvalumab) have been developed to block this pathway, thereby restoring the anti-tumoral immune response. However, the heterogeneity of PD-L1 positivity within the tumor itself as well as across metastatic tumor sites limits its use as a predictive biomarker of response in metastatic BC, with the highest levels seen in the primary tumor and lymph nodes, and the lowest levels seen in the liver [19–22].

IHC is used to quantify PD-L1 protein membrane expression in the TME and is approved as a biomarker of response for ICIs in metastatic TNBC [23]. Multiple IHC scoring methods exist, but the most common is the combined positive score (CPS) generated by the Dako pharmDx assay utilizing the 22C3 monoclonal mouse anti-PD-L1 antibody, which calculates all PD-L1 positive cells (immune and tumor) as a percentage of the total tumor cells and is approved as a companion diagnostic for pembrolizumab in metastatic TNBC. Alternatively, the immune cell (IC) score is determined by the Ventana SP142 PD-L1 assay, which evaluates the percentage of PD-L1 positive immune cells in the tumor area and is approved in some countries to determine eligibility for atezolizumab in metastatic TNBC [24]. The two commonly used cut-offs for PD-L1 positivity are 22C3 CPS $\geq$10 and SP142 IC $\geq$ 1 %, however these scoring systems cannot be used interchangeably given a significant discordance rate [25,26]. Additionally, not all PD-L1 assays have the same sensitivity – the SP142 assay being the least sensitive – and a tumor that is negative for PD-L1 on SP142 could still be positive on an alternative assay [27]. It is not practical, however, for pathology laboratories to implement different assays for the same biomarker.

A combined approach to stratification of the TME based on PD-L1 status and presence or absence of TILs has previously been proposed, with the more immune type I tumors (PD-L1 positive with TILs driving adaptive immune resistance) conferring the best prognosis in a study focused on melanoma [28–31]. Other studies examining this combination in breast cancer subtypes have also suggested that this improves prognostication [32,33]. Further, while not all immune cells stain for PD-L1, the moderate correlation between PD-L1 expression and TILs means that it is possible for tumors to stain negative for PD-L1 despite immune cells being present, thus explaining why PD-L1 negative tumors can still respond to ICIs.

2.1. TNBC

PD-(L)1 monotherapy has limited efficacy in metastatic TNBC; however, patients with immune-enriched tumors demonstrate an increased magnitude of benefit from the addition of targeting agents to cytotoxic chemotherapy [34–38].

Several phase III trials have demonstrated the benefit of utilizing anti-PD-(L)1 ICIs in combination with chemotherapy in patients with metastatic TNBC who are PD-L1 positive (see Table 1). In IMpassion130, the addition of atezolizumab to nab-paclitaxel in patients with untreated TNBC resulted in improved clinical outcomes in the intention-to-treat (ITT) population, with a more pronounced benefit in the PD-L1 positive (IC $\geq$ 1 %) subgroup [39,40]. The presence of sTILs or a basal-like immune activated (BLIA) subtype was also associated with improved progression-free survival (PFS) and overall survival (OS), while patients with both sTIL and PD-L1 positivity seemed to benefit the most [41]. These trial results were not replicated in the follow-up IMpassion131 study, which showed that substituting a paclitaxel backbone plus

Table 1
Analysis of clinical outcomes in key clinical trials that examine the addition of ICIs to chemotherapy in metastatic TNBC, with focus on PD-L1 as a predictive biomarker of response [40–43,73].

Trial	Regime	Population	Median PFS (months)	Median OS (months)
IMpassion130	Atezolizumab + nab-paclitaxel vs placebo + nab-paclitaxel	ITT	7.2 vs 5.5, stratified HR 0.80 (95 % CI, 0.69–0.92, P = 0.002)	21.0 vs 18.7, stratified HR 0.87 (95 % CI, 0.75–1.02, P = 0.077)
		PD-L1 negative (IC <1 %)	Not tested	19.7 vs 19.7, stratified HR 1.02 (95 % CI, 0.84–1.24)*
		PD-L1 positive (IC $\geq$ 1 %)	7.5 vs 5.0, stratified HR 0.62 (95 % CI, 0.49–0.78, P < 0.001)	25.4 vs 17.9, stratified HR 0.67 (95 % CI, 0.53–0.86)*
IMpassion131	Atezolizumab + paclitaxel vs placebo + paclitaxel	ITT	5.7 vs 5.6, HR 0.86 (95 % CI, 0.70–1.05)*	19.2 vs 22.8, HR 1.12 (95 % CI, 0.88–1.43)
		PD-L1 negative (IC <1 %)	Not tested	Not tested
		PD-L1 positive (IC $\geq$ 1 %)	6.0 vs 5.7, HR 0.82 (95 % CI 0.60–1.12, P = 0.20)	22.1 vs 28.3, HR 1.11 (95 % CI, 0.76–1.64)*
KEYNOTE-355	Pembrolizumab + TPC (nab-paclitaxel, paclitaxel or gemcitabine) vs placebo + TPC	ITT	7.5 vs 5.6, HR 0.82 (95 % CI, 0.70–0.98)	17.2 vs 15.5, HR 0.89 (95 % CI, 0.76–1.05)
		PD-L1 positive (CPS $\geq$1)	7.6 vs 5.6, HR 0.75 (95 % CI, 0.62–0.91)	17.6 vs 16.0, HR 0.86 (95 % CI, 0.72–1.04, two-sided P = 0.1125)
		PD-L1 positive (CPS $\geq$10)	9.7 vs 5.6, HR 0.66 (95 % CI, 0.50–0.88)	23.1 vs 16.1, HR 0.73 (95 % CI, 0.55–0.95, two-sided P = 0.0185)

Abbreviations: HR: hazard ratio; IC: immune cell score; TPC: treatment of physician choice; CPS: combined positive score. *Significance not formally tested.

atezolizumab did not increase OS in a similar PD-L1-IC-positive population [42]. This led to the retraction of the accelerated approval of atezolizumab in countries such as the United States and Australia, but it is still available in Europe. Based on biomarker data, it is possible that IMpassion131 may have been positive if sTILs were integrated into patient selection. In contrast, pembrolizumab has been approved for use in combination with chemotherapy in the first-line treatment of metastatic TNBC with PD-L1 CPS ≥10 and is now standard-of-care based on the results of KEYNOTE-355 [43]. A higher cut-off of CPS ≥20 was associated with even greater benefit but not statistically significant, which is similar to what is observed with IC scores (≥1 % vs ≥ 5 %) in IMpassion130 [41,43].

2.1.1. Early setting

Contrary to the findings in metastatic disease, neoadjuvant studies investigating the role of ICIs in early TNBC do not demonstrate a strong role for PD-L1 IHC as a predictive biomarker, though it is associated with improved prognoses regardless of ICI use [44–53].

There is more established evidence for the use of sTILs as a prognostic biomarker in early TNBC, with improved pathological complete response (pCR) rates and long-term disease outcomes independent of chemotherapy and ICIs [16,46,48,54–59]. A retrospective analysis of the adjuvant phase III BIG 02–98 trial, which incorporated docetaxel into anthracycline-based therapy and compared sequential vs concurrent administration of doxorubicin and docetaxel in patients with node-positive BC, showed that sTILs were strongly prognostic for improved disease-free survival (DFS) and OS in patients with TNBC [60]. Analysis of two other large adjuvant phase III anthracycline-containing chemotherapy trials (ECOG 2917 and ECOG 1199) showed that for every 10 % increase in sTILs, there was an 18 % reduction of risk of recurrence (P = 0.02) and 19 % reduction of risk of death (P = 0.01) [54]. Similarly, an increased interval of dynamic change between pre-treatment and on-treatment sTILs with the addition of ICIs to neoadjuvant chemotherapy has been shown to be associated with increased pCR rates, such as in the phase II GeparNuevo study, reflecting the extent of the underlying mechanism of action of ICIs [48,61]. A cut-off of ≥30 % has often been used to identify patients that will have improved clinical outcomes based on this biomarker [56,57]. Indeed, the integration of TILs into clinical prognostic staging using this cut-off has been found to result in up- or down-staging of tumors in a large pooled analysis of patients with early TNBC treated with anthracycline-based chemotherapy in the adjuvant setting [62].

De-escalation strategies for patients with immune-enriched tumors has been an area of ongoing research and presents a promising future direction for biomarker-directed management. There is evidence for the use of anthracycline-free neoadjuvant chemotherapy regimens for patients with early TNBC and high sTILs (≥30 %) as demonstrated by the phase II NeoPACT and Neo-N trials. In NeoPACT (pembrolizumab plus carboplatin and docetaxel), increasing immune enrichment was associated with higher pCR rates, as correlated with sTILs (45 % for sTILS <30 % and 78 % for sTILs ≥30 %) and PD-L1 (40 % with CPS <10 and 74 % with CPS ≥10) [63]. Similarly, in Neo-N (either concurrent or lead-in nivolumab plus carboplatin and paclitaxel) there were higher pCR rates in patients with high sTILs ≥30 % compared to <30 % (66.7 % vs 45.7 %) [64]. Treatment with ICIs alone may also be a future option for this immune-enriched subgroup, as studied in the recent phase II adaptive BELLINI trial which initially enrolled patients with Stage I-III TNBC and high TILs (≥5 %) to receive induction nivolumab (Cohort A) or nivolumab plus ipilimumab (Cohort B) followed by standard-of-care neoadjuvant chemotherapy. Immune activation was achieved in both cohorts, as defined by at least a twofold increase in $CD8^+$ cells on serial biopsy, and clinical response was observed in 12 of 31 patients (38.7 %) who were all found to have TILs ≥30 %. This informed the subsequent opening of Cohort C for patients with node-negative disease and TILs ≥50 % to receive 6 weeks of induction nivolumab plus ipilimumab followed by surgery, with a third of patients achieving pCR with ICI alone that all opted to not undergo adjuvant chemotherapy [65]. In residual disease post-neoadjuvant chemotherapy, higher levels of sTILs are prognostic for improved recurrence-free survival (RFS) and OS, but data is not yet available from a combination approach with ICIs to determine if patients with lower residual cancer burden (RCB) can safely avoid escalated adjuvant treatment [66]. Further de-escalation trials using high TILs to stratify patients to receive different neoadjuvant and adjuvant regimes – such as NeoTRACT (NCT05645380), SCARLET (NCT05929768), ETNA (NCT06078384) and OPTIMAL (NCT06476119) – are also ongoing.

The landmark phase III KEYNOTE-522 trial established the current standard of care for neoadjuvant management of Stage II-III TNBC, with an updated analysis finding that the addition of pembrolizumab versus placebo to anthracycline-based chemotherapy improved 5-year event-free survival (EFS) by 9 % (81.2 % vs 72.2 %) and 5-year OS by 4.9 % (86.6 % vs 81.7 %) with a weight average hazard ratio (HR) for death of 0.66 (95 % CI, 0.50–0.87, P = 0.00150) [64]. While it was initially published that there was an absolute benefit of 13.6 % to pCR rates with pembrolizumab, this was based on an analysis of the first 602 patients, and the final smaller difference of 7.4 % in the overall population is more consistent with the reported OS [67]. PD-L1 IHC did not predict for increased magnitude of benefit, and improved pCR rates with addition of pembrolizumab was seen in patients with both PD-L1 positive tumors (68.9 % vs 54.9 %) and PD-L1 negative tumors (45.3 % and 30.3 %) [52]. Durable improvement in outcomes with pembrolizumab were observed in not only those who achieved pCR, but also in high-risk patients with residual disease, although it is noted that outcomes were still poorer in patients with Stage III disease regardless of pCR, and PD-L1 status remained prognostic in this dataset. Recent exploratory biomarker analysis showed that several biomarkers, including the T-cell inflamed gene expression profile, were not predictive but were positively prognostic for the benefit of pembrolizumab on pCR and/or EFS, but further analysis for sTILs is awaited to help identify a population with very high pCR rates and excellent clinical outcomes to determine which patients may be able to avoid the unnecessary toxicities of the 1-year treatment duration of pembrolizumab. In contrast, it is hypothesized that those with low PD-L1 IHC and low sTILs will likely need additional therapeutic strategies. Other neoadjuvant trials – GeparNuevo, NeoTRIP and IMpassion031 – have also reported improved outcomes with the addition of ICIs to chemotherapy independent of PD-L1 status, though there was a greater numerical benefit in those with PD-L1 positive tumors [48–50]. This guidance in personalizing treatment is much needed given the challenges of limited biomarker data from registrational trials and difficult access to tissue from pharmaceutical-sponsored trials thus far [68].

2.2. Luminal BC

2.2.1. Metastatic setting

ICIs have limited benefit in metastatic luminal BC, with no clear role for TILs as a predictive biomarker of response in the setting [69,70].

2.2.2. Early setting

Early data from two phase III studies – KEYNOTE-756 and CheckMate 7FL – suggests that adding anti-PD-(L)1 ICIs (pembrolizumab or nivolumab respectively) to standard neoadjuvant chemotherapy increases pCR and RCB 0–1 rates in early-stage, high-risk luminal BC and this benefit is particularly seen in tumors with positive PD-L1 IHC or immune enrichment with sTIL >1 % (highest benefit in sTIL >5 %) [26,71]. These rates are comparable to those seen in TNBC and reinforce the strong immunogenicity of some luminal BCs. Further efficacy data is ultimately awaited to determine if these changes in pCR and RCB are associated with improved long-term outcomes as previously described in pooled analysis data, but results may be difficult to clinically integrate in this subtype given that adjuvant CDK4/6 inhibitor inhibition is now standard-of-care [72]. In CheckMate 7FL, greater response with

nivolumab was consistent across both the SP142 (IC ≥ 1 %) and 22C3 (CPS ≥1) assays, though CPS ≥3 was determined as the optimal cut-off for the prediction of benefit given this had the highest overall percentage agreement with IC ≥ 1 % (79.1 %). In the absence of a standardized approach, either sTIL or PD-L1 IHC can be used to select patients with early luminal BC who will benefit most from the addition of ICIs and this can be further validated in future clinical trials. Table 2 compares the pCR in these trials based on the different PD-L1 assays and cut-offs.

2.3. HER2+ BC

2.3.1. Metastatic setting

There is currently limited evidence to support the use of ICIs in metastatic HER2+ BC, though the significant immune infiltration present in this subtype likely mediates signals of improved clinical outcomes when utilizing ICIs to enhance the efficacy of anti-HER2 antibodies and the anti-tumor immune response [4,12]. This was demonstrated in the PD-L1 positive cohorts of the phase Ib PANACEA trial, which treated trastuzumab-resistant patients with pembrolizumab and trastuzumab, and the phase II KATE-2 trial, which randomized patients to TDM-1 plus placebo or TDM-1 plus atezolizumab, with further evaluation planned for a less heavily-pre-treated population in the phase III KATE-3 trial (NCT04740918) [83,84]. Similarly, the retrospective analysis of tumor samples from the phase III CLEOPATRA study highlighted the prognostic value of immune enrichment with sTILs [85]. However, given the association between TILs and PD-L1 IHC positivity, there is doubt as to whether TILs alone add predictive information.

2.3.2. Early setting

There is also insufficient data for ICIs in early HER2+ BC, including in the PD-L1-IC positive population [86]. While several studies have shown that TILs are predictive for pCR after neoadjuvant therapy in HER2+ BC, none have utilized ICIs, and the selection of patients with immune-enriched tumors may provide an additional strategy for future studies in this setting as well as may reduce the use of cytotoxic chemotherapy [87–90]. Patients with residual disease following standard neoadjuvant treatment for HER2+ BC are at high risk for recurrence, and the benefit of adding atezolizumab to adjuvant trastuzumab emtansine will be evaluated in the randomized phase III ASTEFANIA trial (NCT04873362).

Table 2
Subgroup analysis of pathological complete response (pCR) rates in KEYNOTE 756 and CheckMate 7FL [26,71,74].

Trial				pCR	
	Population	Assay score		Anti-PD1 + chemotherapy	Placebo + chemotherapy
KEYNOTE 756	ITT	–		24.3 %	15.6 %
	PD-L1 negative	CPS	<1	7.2 %	2.6 %
	PD-L1 positive	CPS	≥1	29.7 %	19.6 %
			1–9	15.7 %	9.1 %
			≥10	42.3 %	29.0 %
			≥20	53.6 %	36.4 %
CheckMate 7FL	ITT	–		24.5 %	13.8 %
	PD-L1 negative	IC	<1 %	14.0 %	8.2 %
		CPS	<1	14.2 %	10.7 %
	PD-L1 positive	IC	≥1 %	44.3 %	20.2 %
		CPS	≥1	40.4 %	23.8 %
			≥3	53.0 %	25.8 %
			≥5	56.6 %	27.1 %
			≥10	65.7 %	33.3 %
			≥20	78.9 %	26.7 %

Abbreviations: pCR: pathological complete response, PD1: programmed cell death 1, ITT: intention to treat, CPS: combined positive score, IC: immune cell score.

3. Tumor mutational burden (TMB)

TMB is measured by the number of somatic mutations identified per coding region in a tumor genome (mut/Mb). Clinically available assays use next-generation sequencing (NGS) and estimate the TMB based on the genes included in their panel, though the discordance across different diagnostic assays due to factors such as panel size, gene content and the ability to filter out germline variants has led one group to propose statistical calibration of assays through the use of a publicly available software tool to standardize the use of TMB as a biomarker [91–93]. Based on results from two cohorts of the KEYNOTE-158 trial, pembrolizumab has tumor-agnostic approval for tumors considered high TMB (TMB-H) with ≥10 mut/Mb on the FoundationOne CDx™ assay, though notably the subtype was unknown for the five patients with breast cancer that were included [94,95]. Tumors that are TMB-H display a high degree of immune infiltration due to increased neoantigen production and are predictive of patients with improved survival outcomes independent of tumor stage, subtype, treatment and patient age [96,97]. BC has traditionally been traditionally characterized as immune "cold", with a median TMB of 2.63 mut/Mb in one large study of 3969 breast cancer patients, and TMB-H is found in approximately 3.5–5 % cases with higher frequency in TNBC versus luminal tumors and metastatic versus primary tumors [98–100]. The most common mutational signature implicated in genomic instability and TMB-H tumors is apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC), followed closely by mismatch repair deficiency (dMMR) [101]. While APOBEC mutagenesis is associated with lower immunogenicity in certain tumor types, it has been linked to immune activation in breast cancer due to increased activation of $CD8^+$ T cells [102–105].

The efficacy of single-agent pembrolizumab in patients with heavily pre-treated metastatic TMB-H BC was examined in TAPUR, a phase II basket trial which included an arm for 28 patients with metastatic BC that were TMB-H (defined as ≥9 mut/Mb), and KEYNOTE-119, a phase III trial that randomized TNBC patients to pembrolizumab or chemotherapy with an exploratory analysis of 26 patients that were TMB-H (defined as ≥10 mut/Mb). The relatively low overall response rate (ORR) to pembrolizumab in these small patient populations – 21 % in TAPUR and 14.3 % in the TMB-H subgroup of KEYNOTE-119 – suggests a limited role for application of single-agent ICI in TMB-H BC and emphasizes the need for better biomarkers of response or possibly a combination approach with other therapies [106–108]. The latter is supported by exploratory biomarker analysis of KEYNOTE-522, which found a positive association between TMB and pCR in the chemotherapy plus pembrolizumab cohort [109]. Doublet ICI therapy with ipilimumab plus nivolumab in TMB-H patients (defined as ≥9 mut/Mb) was studied in the single-arm NIMBUS trial, with highest benefit seen in in patients with TMB ≥14 mut/Mb (ORR 60 %) compared with TMB ≥9 and < 14 mut/Mb (ORR 4 %), suggesting that a more optimal cut-off could be used to predict benefit [110]. Interestingly, a large retrospective analysis of data from over 10,000 patients by McGrail et al. suggested that TMB-H tumors only derived greater benefit from ICIs in tumor sites where $CD8^+$ T-cell levels correlated with neoantigen load [108].

4. Mismatch repair deficiency

Tumors that are dMMR display high microsatellite instability (MSI-H) and are typically associated with high TMB [99]. dMMR is a predictive biomarker of response to treatment with ICIs, and pembrolizumab has tumor agnostic approval in this setting. However, MSI-H is uncommon in breast cancer and there is minimal data on its predictive value in this tumor type [111].

5. Homologous recombination deficiency (HRD)

A specific mutational landscape characterizes tumors with

homologous recombination deficiency (HRD) due to deficient double-strand DNA. Germline defects in *BRCA1/BRCA2* are the most studied aberration causing HRD and these tumors usually have a two-fold higher TMB than their wild-type counterpart [112,113]. Each responsible gene involved in HRD likely predicts a different sensitivity to ICI therapy. According to one report, *BRCA2* deficient tumors in mouse models have different immune infiltrates and better ICI response than *BRCA1* deficient tumors [114]. A retrospective analysis of patients with metastatic cancers treated with ICIs showed that tumoral *BRCA2* mutation – but not *BRCA1* – was associated with improved OS [114]. However, in IMpassion130, somatic *BRCA1/BRCA2* mutations in metastatic TNBC did not affect PD-L1 IC positivity or outcomes with atezolizumab compared to the overall population [115]. More data about the potential use of HRD as a biomarker for ICI efficacy in BC is needed, particularly in germline and somatic mutations other than *BRCA1/BRCA2*.

6. CD274 gain or amplification

Amplification of CD274 (the gene encoding PD-L1) is rare in solid tumors but could be an independent predictor of response to anti-PD-(L)1 blockade, given it does not always correlate with PD-L1 expression by IHC [116]. In a Chinese study that identified CD274 amplification in 1.09 % of a pan-cancer cohort, there was a demonstrated association between CD274 amplification and other proven biomarkers of response to anti-PD-(L1) ICIs such as TMB, MSI and PD-L1 IHC [117]. In SAFIR02-BREAST IMMUNO, CD274 gain or amplification was associated with an increased OS with durvalumab in all patients, even with controlling for PD-L1 IC IHC ($p < 0.001$) [37]. The role of this possible biomarker of response to ICI needs to be validated in further studies.

7. Estrogen receptor (ER) and/or progesterone receptor (PR) expression

Tumors with ER <50 % have a similar inflamed immune TME to tumors characterized as ER-negative, with higher sTILs, $CD8^+$ cells and expression of immune-related gene sets which are predictive of response to neoadjuvant ICIs. This is reflected in the I-SPY2 trial, where patients with MammaPrint "high" tumors (most likely of luminal B phenotype) had higher rates of pCR with the addition of pembrolizumab to neoadjuvant chemotherapy [118–120]. It is unknown to what extent TILs assessment in I-SPY could have complemented genomics in finding patients with luminal BC that would respond to ICIs. This was studied in the neoadjuvant phase II GIADA trial where patients received three cycles of epirubicin plus cyclophosphamide followed by eight cycles of nivolumab, which found that a combined score of basal subtype and TILs was significantly associated with pCR [121].

Data from exploratory biomarker analysis of CheckMate 7FL also demonstrated the potential role of ER and PR expression by IHC in predicting response when adding nivolumab to neoadjuvant therapy in high-risk, high-grade luminal BC. Higher pCR and RCB 0–1 rates were seen in patients in tumors with low ER (≤50 %) and/or PR (≤10 % in ER ≥ 10 %) [19]. There has been a well-described negative association between ER-positivity and immune infiltrate in BC, with an inverse correlation between the transcriptomic expression of *ESR1* (which encodes ERα) and TILs density, PD-L1 expression and macrophages [70]. Furthermore, increased *ESR1* expression is associated with reduced pCR with use of neoadjuvant ICI in luminal BC [111]. Additional investigation is required in luminal BC to understand the relationship between hormone receptor expression, immune tumoral infiltration, and ICI response.

8. Tumoral immune characterization

Beyond TILs, more precise ways to characterize tumoral immune infiltration have been developed. These include flow cytometry and imaging mass cytometry (IMC), the latter of which enables the simultaneous detection of multiple cell types and proteins via labeled antibodies to reliably assess the spatial interactions with the TME [122]. In NEOTRIP, a study of neoadjuvant chemotherapy with atezolizumab or placebo in early TNBC, analysis of biopsy samples at baseline, after one cycle of neoadjuvant treatment and at surgery was performed using IMC with 43 labeled antibodies. An increase in the density of $CD8^+$ T-cell expressing Granzyme B, a protease released by cytotoxic lymphocytes, during treatment between biopsy samples was predictive of ICI response [123]. While promising, this technique remains investigational and is not routinely available in clinical practice.

9. Gene expression analysis

It is possible to characterize the TME via RNA sequencing (RNA-seq), which utilizes NGS to identify the transcriptome of a tumor cell. Gene expression analysis has played a crucial role in enhancing the understanding of tumor heterogenicity in BC and some groups have developed gene expression profiles (GEPs) to predict response to ICIs [114–116].

In early TNBC, gene expression analysis was conducted as part of the GeparNuevo trial, which studied the addition of durvalumab to neoadjuvant chemotherapy. Several sets of immune genes were evaluated, including the GeparSixto signature (G6-Sig) which was previously demonstrated to be predictive of response to neoadjuvant chemotherapy in TNBC and HER2+ BC, and the IFN signature (IFN-Sig) which was previously shown to be predictive for response to durvalumab in lung and urothelial cancer [55,117]. These two signatures were biomarkers of response to neoadjuvant chemotherapy, but could not discriminate for the benefit of adding durvalumab. By analyzing single genes, they found seven that were involved with interferon (IFN) signaling and cellular antigen processing and presentation, which were significantly associated with pCR in the durvalumab arm but not with placebo (HLA-A, HLA-B, TAP1, GBP1, CXCL10, STAT1, and CD38) [118].

GEPs remain investigational and there has been limited assessment of their clinical utility in comparison to, or in combination with, more simple biomarkers such as TILs and PD-L1, though a recent analysis of 305 patients from the CALGB 40601 and PAMELA studies showed that several B-cell-related signatures were more associated with pCR and EFS than TILs [124].

It is important to acknowledge that different subsets of B- and T-cell immune infiltration have been associated with ICI response, such as $CD8^+$ T_{RM}, high intratumoral $CD8^+$ T-cells or exhausted $CD8^+$ T-cells [113,119–121]. Pathways associated with response or resistance to ICI from gene expression data are summarized in Fig. 1.

An overview of GEPs that have been utilized in clinical trials has been provided in Table 3. Overall, these seem to be highly correlated and this suggests that the signals or pathways being identified are likely to be similar. There is strong potential for many of these to be used in the future, perhaps akin to the current prognostic gene assays used in early stage luminal BC.

9.1. T cell–inflamed GEP ($Tcell_{inf}GEP$)

This signature was developed as a pan-cancer biomarker of response to ICIs and contains 18 IFN-γ-responsive genes [125]. A strong correlation between the $Tcell_{inf}GEP$ and PD-L1 IHC CPS ≥10 was demonstrated in KEYNOTE-086 and, more recently, exploratory biomarker analysis from KEYNOTE-522 found that the $Tcell_{inf}GEP$ was predictive for higher pCR rates and prognostic for improved EFS, independent of ICI administration [109,81]. Further studies are required to demonstrate the value of this GEP as an independent biomarker of response.

9.2. IO score

The IO score, also known as DetermaIO, includes 27 genes related to the immunomodulatory (immune "hot") and mesenchymal (immune "cold") subtypes of TNBC based on a previously established 101-gene

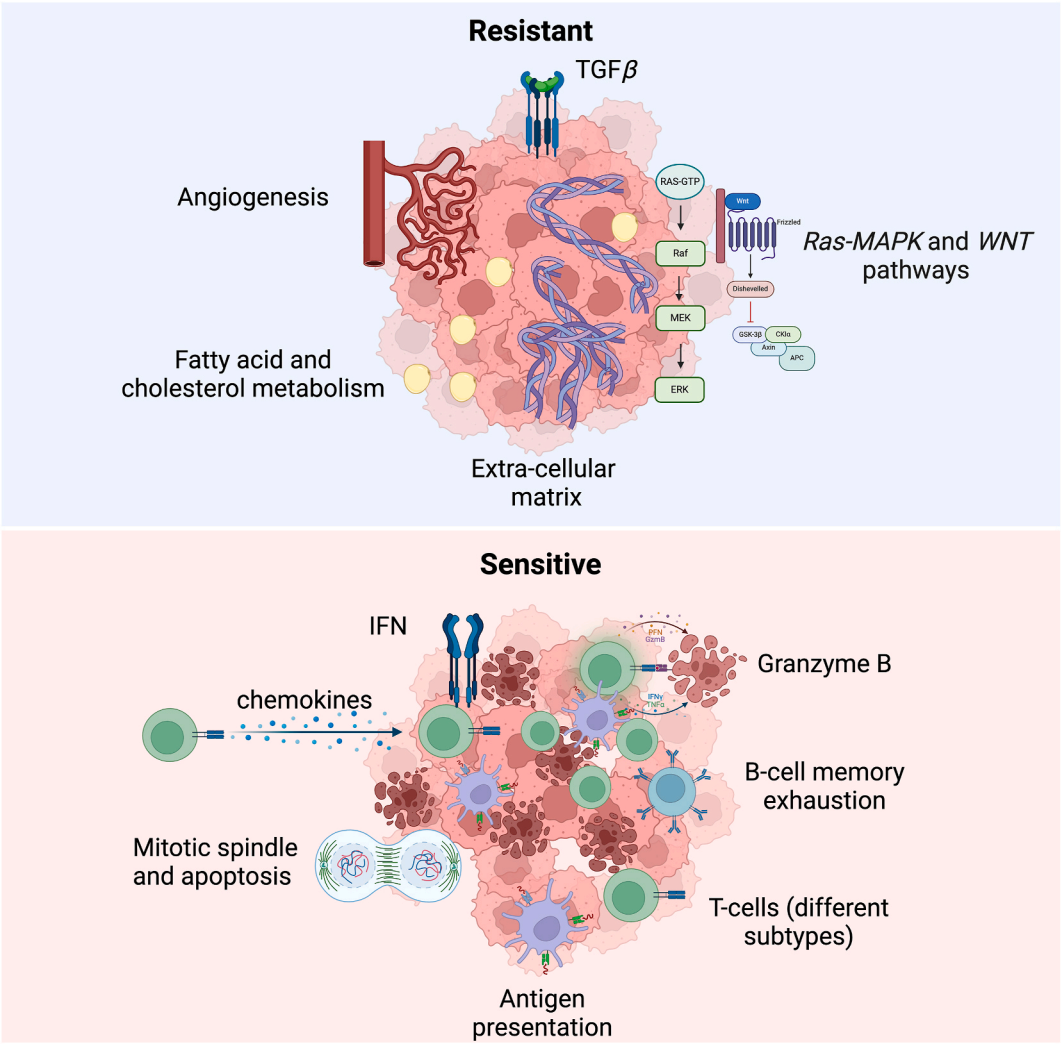

Fig. 1. Schematic representation of different pathways or genes identified via gene expression analysis associated with resistance or sensibility to immune checkpoint inhibitors. *Abbreviations: TGF β: Transforming growth factor-β, MAPK: Mitogen-activated protein kinase, IFN: interferon.*

classification model and has been shown to predict response to ICI in both early and metastatic disease [126]. Initial studies showed the IO score's superiority to PD-L1 IHC in its ability to predict pCR in early TNBC being treated with neoadjuvant therapy [127]. The IO score has shown promising efficacy in predicting ICI benefits in other tumors and overall, larger scale studies are needed [128–130].

9.3. *ImPrint*

The ImPrint score was developed by Agendia and the I-SPY2 consortium by analyzing RNA-seq of pre-treatment early BCs to identify genes associated with pCR to ICIs. This scoring system, comprised of 53 genes predominantly related to immune function, was tested on five arms of the I-SPY2 trial containing ICIs. However, different ICIs and combination therapy with drugs under investigation could have introduced biases, and ImPrint needs to be reproduced in patients treated with the now standard KEYNOTE-522 protocol.

9.4. *TNBC-ICI*

By analyzing publicly available gene expression data, Ensenyat-Mendez et al. used machine learning to develop a GEP comprising 37 genes mainly related to immune function to predict the benefit of neoadjuvant therapy with ICI in TNBC, named TNBC-ICI. A non-statistically significant improved efficacy of TNBC-ICI over the aforementioned IO score in predicting pCR was observed [79].

9.5. *intratumoral CD8$^+$ T-cell signatures*

In TNBC, the presence of high intratumoral CD8$^+$ T-cells is associated with improved outcomes compared to tumors with mainly peripheral or low amounts of CD8$^+$ T-cells (immune "hot" and immune "cold" tumors respectively). Classification of spatial immunophenotypes by these gene signatures can be predictive of benefit from anti-PD1 ICIs in pre-treated metastatic TNBC, independent of PD-L1 expression [82]. High intratumoral CD8$^+$ T_{RM}, which only reside in healthy peripheral tissues and constitute the first line of defense against pathogens, are specifically associated with improved outcomes in BC and offer better prognostication than CD8$^+$ T-cells [131].

10. T-cell receptor (TCR) repertoire

T-cells play a crucial role as effectors of the anti-tumor effects of ICIs and activation is triggered by the binding of the T-cell receptor (TCR) to specific epitopes on major histocompatibility complex (MHC) molecules. During T-cell development, the TCR undergoes multiple somatic recombinations, leading to diverse encoded sequences that determine the epitopes it can recognize. The TCR repertoire, compiled by

Table 3
Gene expression signatures (GEP) tested as a biomarker of response to ICI in breast cancer.

GEP	Trial	Patients	Outcomes
DetermaIO™	NCT02489448 single arm phase I/II NAT + durvalumab in early TNBC [75]	*N* = 55	• Improved OR for pCR (4.13, p = 0.012) with positive IO score
	NEOTRIP phase III randomized trial NAT ± atezolizumab in early TNBC [76]	*N* = 242	• Increased pCR with positive IO score, only with those treated with NAT-ICI (HR: 3.64, p = 0.001) and not NAT alone (HR: 1.31, p = 0.46)
	NEOPACT single arm phase II trial NAT + pembrolizumab in early TNBC [63]	*N* = 115	• Increased pCR with positive compared to negative IO score (72 % vs 42 %, p = 0.002)
	NCT02734290 phase Ib trial NAT + pembrolizumab in metastatic TNBC [77]	*N* = 29	• Improved ORR (43 % vs 29 %) with positive compared to negative IO score • Improved PFS (162 vs. 83 days) and OS (687 vs 305 days) with positive compared to negative IO score • Weak correlation between PD-L1 CPS and IO score
ImPrint	Cohorts of the I-SPY2 trial with ICI for high risk HER2- early BC [78]	*N* = 200 (HR+) + 142 (TNBC)	• In HR+: increased pCR with ImPrint positive (76 %, 44/58) vs negative (16 %, 26/142) • In TNBC: increased pCR with ImPrint-positive (75 %, 54/72) vs negative (37 %, 26/70)
TNBC-ICI	Cohorts of the I-SPY2 trial treated with NAT ± ICI [79]	*N* = 50 (ICI-NAT) + 56 (NAT alone)	• Higher efficiency for predicting pCR in TNBC treated with NAT-ICI compared to NAT alone (AUC 0.86 vs 0.53)
$CD8^+$ T_{RM}	GeparNuevo phase II randomized trial NAT ± durvalumab [80]	*N* = 162	• Excellent outcomes from ICI-NAT compared to NAT alone for DDFS (p = 0.0051) and OS (p = 0.0052)
T-cell inflamed GEP	KEYNOTE 086 phase II trial SA pembrolizumab in metastatic TNBC [81]	*N* = 132	• Improved ORR with higher median expression (19.4 % vs 4.3 %, AUROC: 0.771, p = 0.011) • Higher median expression also associated with improved PFS (p = 0.002) and OS (p = 0.001)
High intratumoral $CD8^+$ T-cells	TONIC adaptive phase II trial of nivolumab after induction treatment in metastatic TNBC [82]	*N* = 53	• Improved OS with $CD8^+$ inflamed signature compared to excluded or ignored (P = 0.05)

Abbreviations: $CD8^+$ T_{RM}: $CD8^+$ T-cell with tissue-resident memory phenotype, DDFS: distant disease-free survival, GEP: gene expression signature, HR+: Hormone receptor-positive, ICI: immune checkpoint inhibitor, N: number of patients, NAT: neoadjuvant chemotherapy, OR: odds ratio, ORR: objective response rate, OS: overall survival, pCR: pathologic complete response, SA: single agent, TNBC: triple negative breast cancer.

sequencing the TCR of peripheral immune cells, is a powerful tool to characterize immune activation and is typically analyzed at multiple timepoints, often from peripheral blood mononuclear cells (PBMCs) [132,133]. The diversity of the TCR repertoire has been suggested as a biomarker of response to ICIs in other types of cancer, such as renal cell carcinoma and melanoma [134,135]. In breast cancer, there has been a correlation between the diversity of TCR repertoire and pCR in patients receiving neoadjuvant therapy without an ICI, with serial RNA-seq at baseline and after two cycles of treatment occurred showing a greater decrease in patients with pCR than those with residual disease [136]. While no study has specifically evaluated the role of TCR repertoire in predicting response to ICIs in BC, this remains a promising area of research, particularly as these could be inferred from peripheral blood draws.

11. Conclusion

The use of ICIs in combination with chemotherapy is approved in TNBC and there is potential to broaden their use to other subtypes, including early luminal and HER2+ BC. The most widely used biomarker – PD-L1 IHC – has a limited role outside of metastatic TNBC and issues arise from the availability of multiple commercial assays with variable levels of concordance. A combined approach of PD-L1 with other biomarkers, including morphological variables such as TILs and other genomic technologies, has previously been proposed but has practical limitations. Recently, the identification of immune-enriched tumors using TILs is emerging as a key strategy across all subtypes to select future trial or real-world populations that may be able to safely de-escalate therapy (such as in early TNBC) or would benefit from the addition of other therapeutic strategies (such as residual disease in early HER2+ BC). High TILs can also select patients that may benefit from the addition of ICIs to neoadjuvant therapy in early luminal BC, alongside low hormone receptor expression which is already routinely evaluated in this setting. While pembrolizumab has tumor-agnostic approval for the TMB-H population, there is low clinical benefit in a BC cohort with single agent ICI and this biomarker may be better applied with a more optimal cut-off to select those who might benefit from doublet ICIs or a combination with chemotherapy. Further, GEPs have shown potential as an important biomarker of response to ICIs, though the use of these is still investigational and therefore less accessible than the more established biomarkers of PD-L1 and TILs. Finally, other novel strategies – such as dMMR, HRD, CD274 gain and amplification, the T-cell repertoire and other methods of tumoral immune characterization – are still under investigation and require further evidence before being integrated into clinical practice.

CRediT authorship contribution statement

Michelle Li: Conceptualization, Writing – review & editing, Writing – original draft. **François Panet:** Writing – review & editing, Writing – original draft, Conceptualization. **Vittoria Barberi:** Writing – original draft. **Roberto Salgado:** Writing – review & editing. **Mafalda Oliveira:** Writing – review & editing, Supervision. **Sherene Loi:** Writing – review & editing, Supervision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2025.104545.

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