# Immune Checkpoint Inhibitors in Triple Negative Breast Cancer: The Search for the Optimal Biomarker

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**ABSTRACT:** Triple negative breast cancer (TNBC) is a high-risk and aggressive malignancy characterized by the absence of estrogen receptors (ER) and progesterone receptors (PR) on the surface of malignant cells, and by the lack of overexpression of human epidermal growth factor 2 (HER2). It has limited therapeutic options compared to other subtypes of breast cancer. There is now a growing body of evidence on the role of immunotherapy in TNBC, however much of the data from clinical trials is conflicting and thus, challenging for clinicians to integrate the data into clinical practice. Landmark phase III trials using immunotherapy in the early-stage neoadjuvant setting concluded that the addition of immunotherapy to chemotherapy improved the pathologic complete response (pCR) rate compared to chemotherapy with placebo while others found no significant improvement in pCR. Phase III trials have investigated the utility of immunotherapy in previously untreated metastatic TNBC, and these studies have similarly arrived at inconsistent conclusions. Some studies showed no benefit while others demonstrated a clinically significant improvement in overall survival in the PD-L1 positive population. It is not yet clear which biomarkers are most useful, and assays for these biomarkers have not been standardized. Given the often serious and severe side effects of immunotherapy, it is important and necessary to identify predictive biomarkers of response and resistance in order to enhance patient selection. In this review, we will discuss both the challenges of traditional biomarkers and the opportunities of emerging biomarkers for patient selection.

**KEYWORDS:** Immunotherapy, immune checkpoint inhibitors, triple negative breast cancer, PD-L1, tumor infiltrating lymphocytes, tumor mutational burden, mismatch repair, cell-free DNA, microbiome, endogenous retroviruses

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## Introduction

Triple negative breast cancer (TNBC) is an aggressive malignancy characterized by the absence of estrogen receptors (ER) and progesterone receptors (PR) on the surface of malignant cells, and by the lack of overexpression of human epidermal growth factor 2 (HER2). TNBC is not amenable to treatment with hormonal therapy or HER2-targeted therapy and likely represents a heterogenous set of cancers. Chemotherapy remains the cornerstone of treatment for TNBC and unfortunately, TNBC continues to be associated with early recurrence and high morbidity despite intervention with chemotherapy. Consequently, prognosis for these patients remains poor in comparison to patients with hormone receptor positive and HER2 positive breast cancers.[1]

However, the therapeutic landscape for TNBC is gradually changing. Sacituzumab govitecan, an antibody–drug conjugate targeting Trop2, which is highly expressed in TNBC, has recently been approved in metastatic breast cancer.[2] In recent years, immune checkpoint inhibitors (ICI) or immunotherapy, has demonstrated favorable outcomes in a variety of refractory solid tumor malignancies including advanced stage non-small cell lung cancer, metastatic melanoma, and metastatic bladder cancer. Unlike these tumor types, most breast cancers are not inherently immunogenic, but TNBC is the most immunogenic of the subtypes of breast cancer for a number of reasons. TNBC has a higher degree of stromal and intratumoral tumor infiltrating lymphocytes (TILs) which recognize and attack tumor cells.[3] Breast cancer has a lower tumor mutation burden (TMB) corresponding to the number of somatic gene mutations present in a tumor, compared to other solid tumors. However, TNBC has a higher TMB than other breast cancer subtypes. Finally, TNBC has been found to have higher rates of cell surface PD-L1 expression when compared to other breast cancer subtypes and higher PD-L1 expression suggests greater potential benefit from the use of PD-1/PD-L1 targeted immunotherapy in this subset of patients.[4-6]

There is a growing body of evidence on the role of immunotherapy in TNBC, however much of the data from clinical trials is conflicting and thus, challenging for the clinician to integrate the data into clinical practice. When considering the results of these trials in TNBC, it is important to distinguish neoadjuvant immunotherapy for early stage disease from immunotherapy for metastatic disease. Tumor immunogenicity, which is the ability of a particular tumor to generate an adaptive immune response, may be influenced by tumor stage. As a tumor grows, cancer cells multiply and accumulate

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**Table 1.** Phase III clinical trials using ICI in early stage triple negative breast cancer.

| TRIAL | REGIMEN | PD-L1 STATUS | PD-L1 TESTING | N | PCR (%) |
|---|---|---|---|---|---|
| KEYNOTE-522 | Paclitaxel and Carboplatin, doxorubicin/ epirubicin + cyclophosphamide +/– Pembrolizumab | + (CPS ⩾ 1) or – (CPS <1 | IHC 22C3 | 602 | 64.8 vs 51.2 (*P* = .00055) |
| NeoTRIPaPDL1 | Carboplatin/nab-Paclitaxel +/– Atezolizumab | + (IC ⩾1% and IC ⩾5%) or – (IC <1%) | SP142 | 280 | 43.5 vs 40.8 (*P* = .66) |
| IMpassion031 | Nab-paclitaxel, doxorubicin + cyclophosphamide +/– Atezolizumab | + (IC ⩾1%) | SP142 | 152 | 69 vs 49 (*P* = .21) |
| | | ITT | | 333 | 58 vs 41 (*P* = .0044) |

hundreds of mutations, leading to the creation of neo-antigens. These neo-antigens may be detected as "non-self" by the adaptive immune system, thereby eliciting an anti-tumor T-cell response. Although all tumors may harbor neo-antigens that can be targeted by T-cells, some tumors express more than others, increasing their immunogenicity, and the likelihood of a robust response to immune-directed therapy.[7,8]

The process of metastasis is thought to involve immune evasion by the primary tumor, which has led to the suggestion that metastatic tumors may be more immunologically inert than early stage tumors. In support of this hypothesis, studies show that metastatic tumors have lower levels of tumor infiltrating lymphocytes (TILs), programed death ligand 1 (PD-L1) protein expression, and mRNA levels of immune related genes than early stage tumors. Each of these factors correspond to lower immunogenicity.[7,8] Conversely, a high tumor mutational burden, is more common in metastatic disease. Somatic mutations are the main source of tumor-specific neoantigens and, therefore, hypermutated tumors would be expected to result in more T-cell infiltration, and improved response to immunotherapy.[8] Given these inherent differences in tumor biology, we will consider the neoadjuvant early-stage trials and the metastatic trials separately.

Three landmark phase III trials tested immunotherapy in the early-stage neoadjuvant setting, KEYNOTE-522 which included 1174 stage II or III patients, and 2 smaller studies, NeoTRIPaPDL1 and IMpassion031 (Table 1). KEYNOTE-522 concluded that the addition of pembrolizumab to chemotherapy improved the pathologic complete response (pCR) rate by 13.6% (64.8% vs 51.2%) compared to chemotherapy with placebo.[9] At the time of submission of this review article, a press release was issued confirming the event-free survival benefit of the addition of pembrolizumab. Similarly, IMpassion 031, in which atezolizumab was combined with chemotherapy resulted in improved pCR rates compared to chemotherapy alone, regardless of PDL1 status.[10] However, NeoTRIPaPDL1, which also evaluated chemotherapy plus atezolizumab in a similar patient population, found no significant improvement in pCR.[11]

Several phase III trials investigated the utility of immunotherapy in previously untreated metastatic TNBC, and these studies have similarly arrived at inconsistent conclusions (Table 2). IMpassion 130 demonstrated no overall survival (OS) benefit with atezolizumab and nab-paclitaxel versus placebo in the overall population, however a clinically significant improvement in overall survival was observed in the PD-L1 positive population (25 vs 18 months).[12] KEYNOTE-355 investigated the addition of pembrolizumab to various chemotherapy backbones, and reinforced the findings of IMpassion 130. It showed a longer progression free survival (PFS) in the pembrolizumab cohort (7.6 vs 5.6 months).[13] On the other hand, the results of IMpassion 131 were unexpectedly disappointing. In this trial, atezolizumab was combined with paclitaxel rather than nab-paclitaxel as in IMpassion 130. PFS was not significantly different in patients who received atezolizumab with paclitaxel versus placebo with paclitaxel in either the intention-to-treat population or the PD-L1 positive population. In addition, there was no improvement in overall survival in either population.[14] Despite the mixed results, the FDA granted accelerated approval to pembrolizumab in combination with chemotherapy in TNBC patients whose tumors express PD-L1 with the IHC 22C3 pharmDx as its companion diagnostic, as well as atezolizumab in the same population with the VENTANA PD-L1 (SP142) assay. At the time of the submission of this review article, the FDA had withdrawn the approval for atezolizumab in metastatic TNBC.

One of the variables that may account for discrepancies in the results of these trials is the differences in biomarkers of response that were utilized, the pitfalls of the assays used to measure these biomarkers, as well as the specific therapeutic antibodies used. Given the often serious and severe side effects of immunotherapy, it is important and necessary to determine which population of patients benefit most from immunotherapy. Clinical investigators have attempted to identify predictive biomarkers of response and resistance in order to enhance patient selection. Nonetheless, it is not yet clear which biomarkers are most useful, and assays for these biomarkers have not been standardized. For this reason, composite biomarkers may be more robust than any single biomarker alone. In this review, we will summarize the major inconsistencies amongst the landmark trials of immunotherapy in the neoadjuvant and metastatic setting, and discuss both the challenges and the opportunities of using biomarkers for patient selection.

**Table 2.** Phase III clinical trials using ICI in metastatic triple negative breast cancer.

| TRIAL | REGIMEN | PD-L1 | PD-L1 TESTING | N | ORR (%) | MEDIAN PFS (MO) | MEDIAN OS (MO) |
|---|---|---|---|---|---|---|---|
| IMpassion130 | Nab-paclitaxel +/– atezolizumab | + or – | SP142 | 902 | 56.0 vs 45.9 | 7.2 vs 5.5 HR, 0.80 (0.69-0.92) | 21.0 vs 18.7 HR, 0.85 (0.72-1.02) |
| | | + (IC ⩾ 1%) | | 369 | 58.9 vs 42.6 | 7.5 vs 5.0 HR, 0.62 (0.49-0.78) | 25.0 vs 18.0 HR, 0.71 (0.54-0.93) |
| KEYNOTE-355 | Chemotherapy (CT) (nab-paclitaxel, paclitaxel, or gemcitabine plus carboplatin) +/– pembrolizumab (P) | CPS ⩾ 1 | IHC *22C3* | CT + P 425<br>CT – 211 | 45.2 vs 37.9 | 7.6 vs 5.6 HR, 0.74 (0.61-0.90) | Results Pending |
| | | CPS ⩾ 10 | | CT + P 220<br>CT – 103 | 53.2 vs 39.8 | 9.7 vs 5.6 HR, 0.65 (0.49-0.86) | |
| IMpassion131 | Paclitaxel +/– atezolizumab | + (IC ⩾ 1%) | SP142 | 292 | 63 vs 55 | 6.0 vs 5.7 HR, 0.82 (0.60-1.12) | 22.1 vs 28.3 HR, 1.12 (0.76-1.65) |
| | | ITT | | 651 | 54 vs 47 | 5.7 vs 5.6 HR, 0.86 (0.70-1.05) | 19.2 vs 22.8 HR, 1.11 (0.87-1.42) |

## Traditional Biomarkers

### *Tumor infiltrating lymphocytes (TILs)*

TNBC is the most likely breast cancer subtype to be lymphocyte predominant (defined as a tumor with >50% lymphocyte infiltrate), and the measurement of TILs has been proposed as a surrogate marker of the adaptive immune response against neoplastic cells.[15] Higher levels of TILs correlate with improved pCR rates in the neoadjuvant setting for all breast cancer subtypes, including TNBC.[16,17] Furthermore, in a pivotal study published in 2010, Denkert et al[18] demonstrated a linear relationship between high levels of TILs and clinical as well as radiologic response to neoadjuvant therapy with anthracycline-based regimens. The results of a 2014 meta-analysis showed that high TILs predicted better overall survival in early TNBC, while a recent systematic review and meta-analysis of 37 retrospective cohort studies revealed that upregulation of TILs predicted higher pCR rates, longer disease-free survival and improved overall survival.[19,20]

Data from KEYNOTE-086, a phase II study, revealed that stromal TIL expression can predict response to immune checkpoint inhibitors. In this study, patients with previously treated metastatic TNBC and any PD-L1 status (cohort A) and patients with previously untreated PD-L1 positive metastatic TNBC (cohort B) received single agent pembrolizumab. Response to therapy was assessed every 9 weeks for 12 months, and every 12 weeks thereafter. For cohort A, the median stromal TIL level in responders was 10% compared to 5% in non-responders.[21] This effect was more pronounced in cohort B in which median stromal TIL level in responders was 50% compared to 15% in non-responders.[22] These findings suggest that, particularly in the first-line setting, TIL levels can predict response to immunotherapy. Data on TILs was not collected in all of the major phase III trials of immunotherapy in TNBC. NeoTRIPaPDL1, though a negative study, did find that pCR rates with atezolizumab were highest in the "immune-rich" group which was defined as either PD-L1 positivity or high/intermediate stromal or intratumoral TILs.[11]

When TILs are broken down into their various subtypes, their effect on immune regulation becomes more nuanced. TILs are made up of CD4+ T-helper cells which facilitate antigen presentation, CD8+ cytotoxic T-cells which are responsible for tumor destruction, and Forkhead box P3 (FOXP3) CD4+ regulatory T-cells which play a key role in generating immunosuppressive T-regulatory cells. CD4+ T-cell and CD8+ T-cells are associated with an improved response to systemic therapy in breast cancer, while FOXP3+ predict a worse prognosis due to their role in facilitating tumor immune evasion.[23-25] Therefore, the ratio of CD8+ to FOXP3 may have more utility than total TILs as a predictive biomarker.[26]

Standardized procedures for quantification and characterization of TILs are lacking, complicating the use of TILs as a potential biomarker. TILs can be measured using semi-quantitative hematoxylin and eosin (H&E) based scores, however this method has low precision and poor reproducibility. To address this challenge, an International Oncology Biomarker Working Group has proposed guidelines for the manual assessment of TILs, which includes separating the stromal compartment from the inter-tumoral compartment, and excluding areas of necrosis and crush artifact.[27] Histologic qualitative scores have also been proposed in which a TIL intensity score is assigned based on the percentage of tumor area infiltrated by lymphocytes.[28] Alternatively, TILs may be more rapidly measured by digital quantification of IHC stained sections using bioimage analysis software such as QuPath.[29] However, such software may be inaccurate if improperly calibrated.[30] An additional challenge to accurate and precise measurement of TILs is intra-tumoral heterogeneity in lymphocyte distribution and limited tumor sampling.[31]

Given the data supporting a correlation of high TIL expression with improved pCR rates and overall survival, optimization of the measurement of TIL expression is necessary for this to be used as a standard biomarker for prediction of response to ICIs. The fact that TILs may be associated with improved response to neo-adjuvant chemotherapy alone may confound attempts to use TILs as predictors of response to combined chemo-immunotherapy regimens.

### *PD-L1 status*

Atezolizumab was initially approved by the FDA in March of 2019 for use in metastatic TNBC, but in order for patients to qualify, their tumors must test positive for PD-L1 expression using the Ventana SP142 assay. This stipulation is based on the findings of IMpassion130, which used this assay as its companion diagnostic, and found that PD-L1 positive patients derived benefit from atezolizumab. In IMpassion 130, median PFS in PD-L1 positive patients was 7.5 months in the atezolizumab plus nab-paclitaxel group versus 5.0 months in the placebo plus nab-paclitaxel group.[12] However, these results were contradicted by the results of IMpassion 131 which also tested the efficacy of atezolizumab in metastatic TNBC using paclitaxel, and which utilized the same assay for PD-L1 testing. In this study, PFS was not improved by the use of atezolizumab in the PD-L1 positive population (6.0 months with atezolizumab vs 5.7 months).[14]

In the neoadjuvant setting, a similar discrepancy was noted between NeoTRIPaPDL1 and both KEYNOTE-522 and IMpassion031. NeoTRIPaPDL1 which utilized the Ventana SP142 assay, a high PD-L1 expression was significantly associated with increased pCR rates.[11] However, in both KEYNOTE-522 (which utilized the IHC 22C3 pharmDx assay) and IMpassion 031 (which used the Ventana SP142 assay), the addition of immunotherapy increased pCR rates independent of PD-L1 status.[9,10]

The inconsistency in data surrounding PD-L1 status and response to immunotherapy may in part be due to poor standardization and reproducibility of PD-L1 expression assays. Each checkpoint inhibitor has its own separate companion diagnostic assay for PD-L1 expression using different antibodies and scoring schemes. Two of the main assays that have been employed in clinical research are the Ventana SP142 assay, which is the companion diagnostic for atezolizumab, and the IHC 22C3 pharmDx assay, the companion diagnostic for pembrolizumab. The SP142 assay is an immunohistochemical assay that uses an anti-PD-L1 rabbit monoclonal antibody that binds to the PD-L1 protein. If at least 1% of the immune cells stain for PD-L1, the tumor is considered PD-L1 positive. The IHC 22C3 pharmDx assay is a qualitative immunohistochemistry assay in which a Combined Positive Score (CPS) is determined by dividing the number of PD-L1 staining cells (tumor cells, lymphocytes, and macrophages) by the total number of viable tumor cells, and multiplying by 100. A tumor with a CPS $<1$ is considered PD-L1 negative, while a tumor with a CPS score of $\geqslant 1$ is considered PD-L1 positive.[32]

The reliability of these assays is somewhat questionable. One study aimed to test concordance in PD-L1 staining in TNBC tissue using the SP142-IHC assay and the IHC 22C3 assay. A total of 135 samples were evaluated, and a total of 62 of those samples had discordant results. Compared to the 22C3 assay, the SP142 assay resulted in underestimation of PD-L1 staining in 53 of those samples, and over-estimation in 9 of those samples.[33] Several other studies also found the 22C3 assay to be more reliable than the SP142 assay.[34] These findings suggest that the use of different reagents often leads to different results and, therefore, these assays are not interchangeable.

In addition to the inconsistency introduced by the use of different assays, there is inconsistency in results even when a single assay is employed. PD-L1 expression is a continuous, rather than a binary, variable which makes it subject to both inter-observer and intra-observer variability. Reproducibility was assessed in a study of 2 sets of 60 non-small cell lung cancer tissue samples using the 22C3 pharmDx assay. The results of this study found that for intraobserver reproducibility, the overall percent agreement (OPA) was 89.7% [95% confidence interval (CI), 85.7-92.6] when using a 1% threshold for determining PD-L1 positivity. For interobserver reproducibility, OPA was 84.2% (95% CI, 82.8-85.5).[35] In another study of intraobserver agreement, 4 different assays were analyzed including 22C3 and SP142. When results were categorized as PD-L1 staining in $<1\%$ or $>1\%$ immune cells, in one third of cases, pathologists found significantly fewer PD-L1 positive tumors with the SP142 assay compared to the other assays, including the 22C3 assay.[34]

There is also limited knowledge regarding dynamic changes in PDL-1expression and intra-tumoral heterogeneity of PD-L1 expression. Sampling time and location of a sample may affect results. Studies have shown that the rate of PD-L1 positivity varies between the primary lesion and metastatic lesions, and PD-L1 positivity also varies with location of the site of metastasis. For example, rates of PD-L1 expression are lower in liver, skin, and bone metastases, and higher in primary lesions, lung, and lymph node metastases.[36] As a result, the site that is chosen for biopsy in metastatic disease may also impact whether a patient's tumor is defined as PD-L1 positive or negative. Each of these factors are likely to further hinder our ability to use PD-L1 expression as a reliable biomarker of response to immunotherapy.

Despite these limitations, data from the clinical trials may suggest certain general trends. One pattern that has emerged is that PD-L1 status seems to be more clinically relevant in the metastatic setting than in early stage disease. Even though the neoadjuvant IMpassion031 and KEYNOTE-522 trials used different PD-L1 assays, both studies showed that pCR rates were higher in patients who received immunotherapy

irrespective of PD-L1 status while the metastatic IMpassion130 and KEYNOTE-355 trials both showed that PD-L1 status predicted response to immunotherapy despite using different assays. These findings support the possibility that PD-L1 status may be irrelevant in early stage disease but significant in advanced disease.

### *Tumor mutational burden (TMB)*

Tumor mutational burden is defined as the total number of acquired, non-synonymous point mutations per coding area in a tumor genome. A high mutational burden is defined at ⩾10 mutations per 1 million base pairs of nucleic acids.[37] Tumors with high TMB generate a greater number of neoantigens, which are recognized by CD8+ T-cells resulting in greater immunogenicity.[38]

High TMB correlates with a higher likelihood of immunotherapy response in tumor types including melanoma and non-small cell lung cancer.[20] Goodman et al conducted a prospective biomarker analysis of the phase II KEYNOTE-158 trial and found that patients with previously treated advanced stage solid tumors had higher response rates to pembrolizumab monotherapy if their tumors had a high TMB. This study involved a myriad of primary tumor types including thyroid, anal, cervical, biliary, and endometrial.[39]

Relatively few studies have been conducted on TMB in breast cancer. However, one study which combined data from 6 publicly available genomic studies found higher TMB in metastatic tumors compared to primary tumors (3.8 vs 2.0), and a significantly higher median TMB in TNBC (1.8 mut/Mb) compared to HR-positive (1.1 mut/Mb) or HER-2 positive (1.3 Mut/Mb) cancers. This same study also determined that the hypermutated breast cancers had a higher neo-antigen burden.[21] Based on this data, we might expect metastatic TNBC to have higher TMB than other subtypes and, therefore, be more immunogenic; although the absolute TMB level is still low. Furthermore, tumors with deficient mismatch repair (dMMR), microsatellite instability (MSI), Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) dysregulation, or DNA polymerase epsilon (POLE) mutations, tend to have high TMB.[40-43]

DNA polymerases are responsible for recognizing and removing errors that occur during the replication process. POLD1 and POLE are the specific polymerases required for removing errors in lagging and leading strand DNA synthesis, respectively.[44] Somatic loss of function mutations in the genes for these polymerases result in high TMB.[45] In a study of 47 721 patients with different cancer types, POLD1/POLE mutations were identified in 94 of 657 breast cancers.[46] This study also assessed response to immune checkpoint inhibitors in POLE and POLD1 mutated patients. They found that treatment with ICIs was associated with a significantly longer OS of 34 months in patients with POLE/POLD1 mutations compared to 18 months in the wild type population. When cancer type and MSI status were adjusted for, POLE/POLD1 mutations was an independent predictor of response to ICI treatment.[46]

RNA editing plays a substantial role in tumor hypermutation. The APOBEC gene signature is comprised of cytidine deaminases, enzymes that are capable of converting cytidine (C) bases at specific positions of RNA to uridine (U) bases. In one study of 3969 breast cancer patients' samples, 59.2% of hypermutated breast cancers (defined as >10 mut/Mb) had a dominant APOBEC activity signature.[40] APOBEC-3 mediated C to U editing is particularly prevalent in breast cancer tumors.[47] APOBEC upregulation correlates with high levels of PD-L1 expression.[48] In NSCLC, the APOBEC mutational signature is specifically enriched in the population of patients who have a durable response to ICI treatment.[49]

There is clinical data to support a correlation between TMB and pCR rates in TNBC. Karn et al[50] calculated TMB in 149 TNBC pre-treatment samples from GeparNuevo, a phase II neoadjuvant trial that showed the addition of durvalumab to chemotherapy increased the pCR rate in early stage TNBC. They found that median TMB was significantly higher in the tumors of patients who achieved pCR than in those who did not (1.87 vs 1.39 Mut/Mb, $P = .005$). In addition, the TAPUR study demonstrated response to single agent Pembrolizumab in 28 metastatic breast cancer patients with high TMB defined as ⩾9 Mut/Mb. In this study, Pembrolizumab given every 3 weeks resulted in objective response in 6 patients (21%), with a median PFS of 10.6 weeks and a median OS of 31.6 weeks.[51]

Tumor mutational burden can be challenging to measure. Tissue from a primary or metastatic lesion is prone to sampling bias due to tumor heterogeneity.[52] Plasma TMB from a liquid biopsy may be a more complete representation of the mutational landscape, but further studies are needed to establish the role of TMB from liquid biopsy.[52] The computational methods used to quantify plasma TMB differ from those used to quantify tissue TMB, therefore they are not directly equivalent to one another. Plasma TMB evaluated only single nucleotide variants, while tissue TMB is also able to account for indels and fusions.[53] Another challenge in effectively utilizing TMB as a biomarker is that different thresholds, in terms of number of mutations per megabase, have been used to define high TMB. The various assays that are available also differ in the number of genes analyzed and the types of mutations that are incorporated into the analysis.[54] Measuring challenges of TMB and the fact that it is not as elevated in TNBC compared to other tumor types, make it difficult to establish it as a reliable biomarker.

### *Mismatch repair (MMR)*

Mismatch repair (MMR) refers to a biological system responsible for recognizing and repairing mismatched base pairs that arise during DNA replication. The 4 proteins involved in MMR

are MLH1, MSH2, MSH6, and PMS2. Mutations in any of these genes result in genomic instability and, ultimately, predisposition to cancer.[55] The development of antibodies to the MMR proteins has allowed us to use immunohistochemistry to detect MMR deficiency.[56] MMR deficient tumors are associated with a high TMB.[45] Microsatellite Instability (MSI) refers to a phenotype that is associated with MMR deficiency. Microsatellites are short, repeated sequences of DNA bases. Cells with MMR deficiency are unable to correct errors that occur during replication which results in the formation of novel microsatellites in the DNA. Polymerase chain reaction assays can identify these novel microsatellites, and allow us to assess for microsatellite instability in tumors.[57] The FDA granted approval for pembrolizumab in any MSI-high or MMR-deficient metastatic solid tumor that has progressed on prior treatments. This approval is based on evidence from KEYNOTE-158 which showed that MSI-high tumors are susceptible to immune checkpoint inhibition, with a particularly robust response rate in colorectal, GI, and endometrial cancers.[58]

Compared to other tumors types, it is uncommon for breast cancers to be MSI-High. In a study that included 922 breast cancer samples, only 1.7% were MSI-High.[59] In a separate study of 316 breast cancer specimens, only 4 ($<$2%) were MMR deficient, and interestingly all 4 were in the triple-negative group.[60] Breast cancers that do harbor MMR deficiencies have been found to positively correlate with patients' survival.[61] Despite this, there is data to suggest that MMR deficiency detected by IHC does not always correlate with MSI as determined by pCR assays. In a study by Fusco et al,[61] 75 dMMR breast tumors were evaluated for MSI status. 91% of the dMMR tumors were microsatellite-stable. In addition, intratumor heterogeneity was noted, most commonly in expression of the MSH6 protein. These findings indicate that a single biopsy may have questionable clinical value, particularly when small samples such as core biopsies are involved. Furthermore, MMR analysis by IHC is not interchangeable with MSI analysis.

There are no large clinical trials of the relationship between dMMR and response to immunotherapy, and there are very few case reports in the literature of dMMR breast cancer patients who have had a robust response to immunotherapy.[62] Given the problem of intra-tumoral heterogeneity, breast cancer specific biomarkers have been sought. PTEN has been proposed as a potential complementary biomarker. PTEN is a tumor suppressor through negative regulation of the PI3K/Akt signaling pathway. This pathway is responsible for preventing cell growth and proliferation by allowing for the expression of proapoptotic factors.[63] PTEN protein expression by IHC has been linked to a high likelihood of intact expression of all 4 MMR proteins with a Positive Predictive Value of $\geqslant$94%.[64,65] In one study, 82% (66/81) of dMMR breast cancers had low levels of PTEN expression or a decreased number of copies of the gene. 95% (313/328) of breast cancers with retained PTEN expression were MMR proficient or heterogeneous.[65] Although the mechanism behind the correlation between PTEN expression and MMR status remains unclear, given the high Positive Predictive Value, PTEN is considered a potential first-line screening test to aid in identification of MMR proficient tumors. Interestingly, unlike expression of MMR proteins by IHC, PTEN expression has been found to be homogenous within tumor samples.[65]

## Emerging Biomarkers of Sensitivity to Immunotherapy

### *Role of the microbiome in immunotherapy*

Microbiota refers to the bacteria that inhabit the GI tract while microbiome refers to the collective genome of an individual's microbiota. The composition of microbiota is dynamic and modulated by host genetics as well as external factors such as diet, medications, and environmental toxins.[66] Two metrics have been outlined to describe the complexity and diversity of microbiota—alpha-diversity which describes the number of organisms and the uniformity of their distribution, and beta-diversity which describes the degree of overlap and variance in microbiota from different sites.[67] Microbial pathogens, such as *H. Pylori* and *Schistosoma Haematobium*, are known to be drivers of carcinogenesis. In addition, certain species have been found to be more and less prevalent in different tumor types. In breast cancer, the microbiota composition is enriched in *Bacillus, Enterobacteriaceae*, *Staphylococcus*, and unclassified *Bacteroidetes*.[68]

Microbiota are intimately connected to the development and function of the immune system. For instance, they have been shown to regulate neutrophil migration, T-cell differentiation, and expansion of the regulatory T-cell population.[69,70] Given the close interaction between the microbiome and host immunity, there has been considerable interest over the past decade in exploring ways in which the microbiome can be harnessed to improve tumor response to immunotherapy. In mouse models of melanoma, differences in the microbiome were linked to tumor response to anti-PD-L1 therapy. Furthermore, fecal microbiota transplant from the more responsive mouse line to the less responsive mouse line enhanced anti-PD-L1 efficacy. In this study, *Bifidobacterium* was identified as the crucial bacteria because "therapeutic feeding" of this single organism was able to enhance response to PD-L1 inhibition.[71] In another mouse model study of anti-CTLA-4 immunotherapy, anti-CTLA-4 therapy had no significant effect on tumor burden in the germ-free state while, introduction of *Bacteroidales* and *Burkholderiales* into the host microbiome resulted in a reduction of tumor burden.[72]

There is mounting evidence of clinical data showing that the human gut microbiome is associated with response to anti-PD-1 and PD-L1 immunotherapy in a number of solid tumors including melanoma, non-small cell lung cancer, gastrointestinal cancers, and hepatocellular carcinoma.[67,73-76] Although data

in breast cancer is limited, one study investigating the human microbiome of 30 patients with TNBC. Swoboda et al[68] was able to identify *Bacteroides* and *Ruminococcaceae* as species that were more abundant in TNBC patients who achieved a pCR after treatment with neoadjuvant chemotherapy compared to those who did not achieve a pCR. Amongst patients who had residual disease, those with a partial response had greater quantities of Bacteroides caccae than those who had no response. To date, there is no data on whether the composition of microbiota affects response to immune checkpoint inhibition in breast cancer patients. Given the favorable data in other tumor types, this is worthy of further investigation.

## *Chemosensitivity as a marker of immunotherapy sensitivity*

Cytotoxic chemotherapy is the backbone of systemic treatment in TNBC. The standard of care in the neoadjuvant setting is sequential anthracycline and taxane based therapy. Chemotherapeutic agents can induce various immunomodulatory changes that prime the tumor microenvironment to increase responsiveness to immunotherapy. For instance, chemotherapeutic agents upregulate PD-L1 expression, increase expression of immunogenic cell surface markers such as MHC1, and increase release of neo-antigens by tumor cells.[77-79]

Different chemotherapy agents enhance immunogenicity through different mechanisms, and certain modifications may have a greater effect on immunogenicity than others. Treatment with taxanes such as paclitaxel have been shown to increase recruitment of TILs while docetaxel decreases immunosuppression, thereby allowing a greater anti-tumor immune response by decreasing T-regulatory cells and myeloid-derived suppressor cells (MDSCs).[80-82] Treatment with platinum agents promotes T-cell activation, increases expression of MHC class I antigens on cancer cells, and downregulate MDSCs.[83] Treatment of tumor-bearing mice with anthracyclines in pre-clinical trials resulted in increased CD4+ and CD8+ T-cell infiltration and reduced intra-tumoral MDSCs which play an integral role in inhibiting anti-tumor immunity.[84] Despite all this evidence however, the positive neoadjuvant clinical trials, KEYNOTE-522 and Impassion 031 show almost similar contributions to pCR by ICI regardless of the chemotherapy agents used, so it is unclear at this time that choice of chemotherapy backbone may be used to enhance ICI sensitivity.

## *Cytoplasmic DNA and the cGAS-STING pathway*

DNA is normally found in the nucleus or mitochondria of human cells. DNA located in the cytoplasm is the result of a microbial infection or DNA damage, such as that which occurs in cancer, and a signal that can activate the innate immune system. Cyclic GMP-AMP synthase (cGAS) is a sensor that recognizes cytoplasmic DNA. The binding of cGAS to double-stranded DNA (dsDNA) activates the cyclic GMP–AMP synthase—stimulator of interferon genes (c-GAS- STING) pathway. Activation of this pathway ultimately results in anti-proliferative cell states, including cellular senescence and early apoptosis.[85] In certain settings, activation of this pathway may also have a pro-tumorigenic effect. Inactivation of the BRCA2 gene, which increases the likelihood of developing breast cancer, impairs DNA production and the accumulation of micronuclei in turn, activates the cGAS-STING pathway. This activation causes cell cycle arrest and triggers apoptosis.[86] However, if BRCA2 is chronically or permanently inactivated, resulting in perpetual activation of the cGAS-STING pathway, cell cycle progression is restored, allowing for survival of mutated cells.[87] Cell-free DNA (cfDNA) refers to segments of DNA released into the bloodstream when cells and broken down and release their contents. Cancer generates high levels of cfDNA in a patient's serum due to increased cellular necrosis and apoptosis driven by tumor cell division.[88] Consequently, cfDNA is considered a surrogate marker for activation of the cGAS-STING pathway.[89]

The cGAS-STING pathway is essential for the antitumoral activity of ICIs. Mice that are deficient in this pathway are resistant to the effects of PD-L1 inhibition.[90] In addition, the combination of STING agonists with immunotherapy demonstrated synergistic anti-tumoral effects.[91] Taking advantage of cfDNA-mediated activation of the STING-cGAS pathway may be a promising method for optimizing immune checkpoint inhibitor therapy, and cfDNA may have utility as a biomarker in this setting. The benefit of such a biomarker is that tumor tissue biopsy would be unnecessary since cfDNA can be easily collected as a circulating liquid biopsy biomarker.

## *Endogenous retroviruses*

Endogenous retrovirus (ERVs) are remnants of exogenous infectious retroviruses that have been integrated into the germline and they make up about 5% of the human genome.[92] ERVs are silenced in normal cells, but demethylated and re-expressed in tumor cells.[93] They have low or undetectable expression in normal tissue cells, and are overexpressed in tumor cells including in breast cancer cells, in particular.[94,95] Expression of ERVs is associated with response to immune check point inhibition, specifically in cancers with low mutation burden such as natural killer-lymphoma and Hodgkin's disease.[96] Response to immunotherapy has also been correlated with expression of ERVs in urothelial cancer and clear cell renal carcinoma.[97] This correlation is thought to be related to the ability of ERVs to act as tumor-specific antigens, capable of inducing an immune response.[98] One study found that ERVs in breast cancer cells induce a cytotoxic T-cell response targeted to the ERV env protein, and that these cytotoxic lymphocytes are able to lyse cancer cells expressing this target.[99] Measurement of ERV RNA expression using PCR may, therefore, be a potential biomarker for response to immunotherapy in triple negative breast cancer.

### *Tumor neoantigen load*

During tumorigenesis, cancer cells acquire genetic alterations. Tumor-specific mutated genes encode neo-antigens which are then expressed, processed, and presented on tumor cell surfaces. These neoantigens are recognized by T-cells. Normal cells do not acquire these somatic mutations and, therefore, are not subject to destruction by neoantigen-specific T-cells. As a result, these neoantigens are potential targets for T-cell based immunotherapy.[7] Neoantigen expressions has been proposed as a potential biomarker to predict response to immunotherapy.[100] Interestingly, TMB does not necessarily have a linear correlation with neoantigen load, as might be expected. Not all mutations result in the generation of neoantigens. Mutations in certain critical loci may be more likely to generate neoantigens than mutations at other loci, providing a potential explanation for why certain tumors with low TMB still respond well to immunotherapy.[101]

### *Composite biomarkers*

The power of any single biomarker to accurately predict response to immunotherapy is limited by variability in assays, spatial and temporal heterogeneity, and inter-reader reproducibility. Accuracy may, therefore, be increased by utilizing a composite biomarker comprised of multiple predictive factors. A study of advanced NSCLC by Rizvi et al,[102] for example, found that while PD-L1 status and TMB were independent biomarkers of response to immunotherapy, patients whose tumors were PD-L1 positive and had a high TMB had the highest rate of durable clinical benefit (DCB), defined as complete response, partial response, or stable disease that lasted >6 months. PD-L1 expression (using various antibodies including 22C3 and E1L3N), did not correlate with TMB, suggesting that these biomarkers identified distinct populations. When considered as a composite variable, patients with both high TMB and PD-L1 positivity had a DCB rate of 50%. This was higher than the DCB rate seen when only one of these variables was present (35.3% for positive PD-L1 expression but low TMB and 29.4% for high TMB but negative PD-L1 expression.[102] In NSCLC, Yu et al[103] found that a composite of 3 predictive markers, CD8+ TIL, TMB, and PD-L1 expression, was associated with longer OS and PFS compared to any of these markers alone or to any 2 of the 3 biomarkers combined. Similarly, Althammer et al[104] found that NSCLC patients with both high PD-L1 expression and high TIL density had the longest PFS when treated with a PD-L1 inhibitor.

These findings support the possibility that a composite score incorporating both TMB and PD-L1 status may be a more useful biomarker than either of these variables alone. Integrating data from different components of tumor biology and host factors may, therefore, prove to be a more robust predictor of response to immunotherapy than any single factor alone.

## Conclusion

Triple negative breast cancer is a highly heterogenous disease and continues to harbor a particularly poor prognosis compared to other breast cancer subtypes. Immune checkpoint blockade has emerged as a promising therapeutic option for some TNBC patients. However, clinical trials have demonstrated mixed results with respect to response of TNBC to immune checkpoint inhibition. We are currently unable to predict with precision which TNBC patients will respond optimally to immunotherapy. It is well known that exposure to immunotherapy is associated with the risk for significant adverse events. Learning how to best select patients who are suitable for immunotherapy is imperative, especially in early stage breast cancer where the goal is for curative intent and the risk of long-term adverse events from immunotherapy may be debilitating. Reliable and reproducible biomarkers must be identified in order to achieve this goal.

PD-L1 expression has not consistently correlated with response to immunotherapy in clinical trials, and therefore cannot yet be considered a meaningful predictive biomarker. The different antibodies used in immunohistochemical tests, the disparity in expression in primary and metastatic site biopsies, and the potential for intra-tumoral heterogeneity are unresolved obstacles to reproducibility and reliability. TMB and TILs are being explored as PD-L1 independent predictive biomarkers. However, methods for measuring TMB and TILs require further standardization and validation. These traditional biomarkers present a number of challenges which may be difficult or even impossible to overcome.

Promising novel biomarkers are emerging. There is encouraging evidence of the role of the microbiome in predicting response to immunotherapy. While the role of the gut microbiome has been studied in the pre-clinical setting and has been found to be associated with response to anti-PD-1 and PD-L1 immunotherapy in a number of solid tumors, breast cancer specific data is lacking. Similarly, analysis of expression of ERV and repetitive RNAS, and use of rearrangement-induced neoantigens may also be informative of response to ICB in TNBC, given that these cancers have a high burden of genomic rearrangements and epigenetic alterations. cfDNA may prove useful as a liquid biopsy biomarker in predicting response to immunotherapy, and would be a simple and non-invasive biomarker to measure. Although traditional tumor biomarkers such as TMB, TILs, and PD-L1 status may be suboptimal individually, combining multiple biomarkers into a composite score may be more efficacious.

There is currently insufficient clinical data regarding the real-world utility of various potential biomarkers in predicting response to immunotherapy. There are, however, a few active clinical trials that seek to address this issue. The PARADIGM trial is currently recruiting participants for an observational study assessing the association between the gut microbiota and ICI treatment efficacy in multiple solid tumor types, including breast

cancer.[105] In addition, ADIGYN is a large prospective cohort study designed to evaluate the role of ctDNA in predicting resistance to oncologic treatments in digestive and gynecologic/breast cancer. This study is currently recruiting. It is not, however, specific to immunotherapy.[106] Finally, there is an ongoing study of cell free DNA assay as a potential biomarker for predicting early non-response to therapy (including immunotherapy) in metastatic cancer non-small cell lung cancer, colorectal cancer, and breast cancer.[107] There are no registered clinical trials currently investigating composite biomarkers, TMB, TILs, tumor neoantigen load, or ERVs as predictive biomarkers.

As novel predictive biomarkers are being developed, vigorous criteria must be applied. This includes ensuring that the biomarker under investigation is associated with overall survival across multiple independent clinical trials. This is pertinent as there are a number of new investigational drugs in combination with ICIs currently ongoing in clinical trials. It will require that the biomarker be highly reproducible, and that it be measured using a single, standardized assay. Further research in this area will lay the groundwork for tailoring immunotherapy in TNBC.

## Author Contributions

CO conceived the idea, wrote and revised the article. SQ wrote and revised the article. NC, MG, SG, DT were involved in manuscript revisions and all authors approved the final version for publication.

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