ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

ANNALS OF ONCOLOGY driving innovation in oncology

REVIEW

The journey of tumor-infiltrating lymphocytes as a biomarker in breast cancer: clinical utility in an era of checkpoint inhibition

S. Loi[1,2*], S. Michiels[3,4], S. Adams[5], S. Loibl[6,7,8], J. Budczies[6,9], C. Denkert[10] & R. Salgado[1,11]

[1]Division of Research, Peter MacCallum Cancer Centre, Melbourne; [2]Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville, Australia; [3]Department of Biostatistics and Epidemiology, Gustave Roussy Cancer Campus, University Paris-Saclay, Villejuif; [4]Oncostat INSERM U1018, labeled Ligue Contre le Cancer, University Paris-Saclay, Villejuif, France; [5]Perlmutter Cancer Center, New York University School of Medicine, New York, USA; [6]Institute of Pathology, Heidelberg University Hospital, Heidelberg; [7]German Breast Group, c/o GBG Forschungs GmbH, Frankfurt; [8]Goethe University, Frankfurt; [9]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg; [10]Institute of Pathology, Philipps-University Marburg and University Hospital Marburg (UK-GM), Marburg, Germany; [11]Department of Pathology, GZA-ZNA Hospitals, Antwerp, Belgium

Available online 24 July 2021

In 2014, we described a method to quantify percentage of tumor-infiltrating lymphocytes (TILs) on hematoxylin and eosin-stained slides of breast cancer samples using light microscopy that could be performed easily by pathologists with no extra stains. The aim of detailing the method was to facilitate independent research groups replicating our prognostic findings using TIL quantity in early-stage breast cancers. A global working group of breast pathologists was convened to standardize, test reproducibility, and refine the method. A website was also established which allowed free training (www.tilsinbreastcancer.org). As a result of this work, TIL data have been collected in over 20 000 primary breast cancer samples worldwide and the robust associations with better prognoses in triple-negative breast cancer (TNBC) and HER2+ BC have been confirmed. This has resulted in the inclusion of the TIL biomarker in several international breast cancer guidelines as well as in national criteria for routine pathology reporting. TIL therefore represents the first biological prognostic biomarker for early-stage TNBCs, and here its prognostic effect is linear, with values of 30%-50% being suggested as suitable for use in potential chemotherapy de-escalation studies. The efficacy of immune checkpoint-targeted agents in breast cancer now provides direct evidence that host immune responses can modify tumor growth in some patients. With the recent granting of accelerated approvals for the first PD-1/PD-L1 targeting agents in early and advanced TNBC, our focus has now moved to investigating the clinical utility of TIL in the setting of immune checkpoint agents, with or without PD-L1 protein assessment. Emerging data suggest that TIL quantity can help clinicians identify patients with breast cancer who benefit most from PD-1/PD-L1 inhibition. In patients with advanced TNBC and HER2+ disease a TIL cut-off of 5% or 10%, with PD-L1 expression can define 'immune-enriched' tumors and currently seems to have the most clinical relevance in this context.

Key words: tumor-infiltrating lymphocytes, breast cancer, triple-negative breast, cancer, checkpoint inhibitors

INTRODUCTION

The risk factors for breast cancer in the general population are largely hormonal related. Given its incidence is not increased in patients on chronic immunosuppression, the growth and progression of this breast cancer have not traditionally been thought to be susceptible to host immunosurveillance. Immune infiltrates have long been noted in breast cancer samples[1]; however, their clinical relevance has not been clearly elucidated until the observation that breast cancer is at least three different biological subtypes.[2] In the highly proliferative subtypes, usually the triple-negative and HER2-overexpressing breast cancers, immune infiltrates are most frequently seen.

To evaluate the clinical relevance of immune infiltration, we developed a method to quantify breast cancer lymphocytic infiltration that was pragmatic, straightforward, and easy to apply using standard hematoxylin and eosin (H&E) slides of diagnostic breast cancer samples. Our hypothesis at that time was that a simple method would increase uptake by pathologists, as well as facilitate the

*Correspondence to: Prof. S. Loi, Peter MacCallum Cancer Centre, 305 Grattan St., Melbourne, Victoria 3000, Australia; Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville, Australia. Tel: +61 3 85595935; Fax: +61 3 85595039

E-mail: sherene.loi@petermac.org (S. Loi).

generation of data that would help us understand the relationship between the immune system and breast cancer growth, as well as its potential prognostic and predictive biomarker capabilities. Quantification of immune markers using gene expression, single and multiplex immunohistochemistry (IHC), and new technologies such as digital spatial profiling and single-cell analyses now also confirm the strong role the immune system plays in outcomes of some breast cancer subtypes. However, many immune markers that are indicative of a T-cell antitumor response are strongly correlated, and while qualitative differences in the immune infiltrate (i.e. how specific immune subsets relate to survival and therapeutic outcomes) are being intensely investigated, it remains surprising that a simple measurement of tumor-infiltrating lymphocyte (TIL) quantity using a microscope or a digital whole slide image continues to provide strong prognostic value. Hence evaluation of TIL could be a less expensive and valuable alternative or partner to many of these newer emerging technologies as we move forward into the era of immunotherapy. In this article, we summarize how we standardized and disseminated our TIL biomarker methodology, as well as the efforts to understand its relevance in breast cancer biology and for patients.

DEVELOPING TECHNICAL AND ANALYTICAL STANDARDIZATION AND REPRODUCIBILITY OF THE TIL BIOMARKER

Pathologists are required to provide reliable and reproducible data on quantitative tissue biomarkers, particularly in cancer where the clinician is heavily reliant on the right diagnosis, molecular alteration as well as quantification of druggable targets in order to prescribe the correct treatment for patients with cancer. In order to learn from past lessons of other cancer biomarkers such as estrogen and progesterone receptor quantification, HER2 IHC, histological grade, and lately, Ki67, the International Immuno-Oncology Biomarker Working Group (www.tilsinbreastcancer.org), also called the TIL working group (TIL-WG), sought to ensure that the TIL quantification method could be standardized across pathologists in different laboratories globally. In order to do this and to reduce both systematic and random variation among reporting, we developed a training tool that recapitulates how pathologists think, namely, by remembering reference images of specified TIL levels, similar to how they do for Ki67 or hormone receptor staining, where images selected by pathologists are compared with a set of reference images, allowing direct visual comparison of the individual image. Then, whenever the pathologist encounters different zones in tumor, with different TIL densities, these reference images allow him/her to assess the TILs for each zone, and afterward a mean of the fields is calculated, which is the final TIL score, similar to how it is done for other biomarkers in breast cancer daily practice (Figure 1). Training materials are freely available on the website www.tilsinbreastcancer.org, including a short movie detailing each step of TIL analysis.[3]

This resulted in an excellent interobserver concordance for stromal-based TILs, with an intraclass correlation up to 0.86,[4] and this compares favorably with the reproducibility between pathologists of other commonly used morphological features in routine daily breast cancer practice.[5,6] Among patients with ductal carcinoma *in situ*, we found that TIL evaluation in this carcinoma was highly reproducible with an intraclass correlation coefficient of 0.96.[7,8]

We have subsequently refined this methodology for metastatic organs and lymph node samples, and we have also defined the method for use in samples from other solid tumors. The method is now used in at least seven other different tumor types, including lung cancer and colorectal cancer.[9]

CLINICAL VALIDITY AND UTILITY OF TILS IN BREAST CANCER

The advantages of a biomarker that can be used without extra stains and can be utilized on archival formalin-fixed paraffin-embedded (FFPE) tissue using light microscopy are immense. The use of large datasets with long-term follow-up, particularly from randomized clinical trials, allows researchers to potentially answer questions about prognosis as well as interactions with the treatment effect of chemotherapy and immune checkpoint inhibition. Samples collected from patients enrolled into randomized clinical trials are of high value as they are collected in a standardized manner: patients receive controlled treatments and thus can provide information on the patients who benefited from the investigational treatment. While prospective incorporation into clinical trials remains the top level of evidence to conclusively define a biomarker's predictive value, retrospective analyses of prospective phase III large randomized clinical trials can also be very informative and provide level IB-evidence.[10]

Prognostic and predictive value of TIL in patients with breast cancer undergoing standard treatments

Collectively (Table 1; Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2021.07.007), the data show that higher quantity of TIL has strong prognostic associations, particularly in the setting of early-stage triple-negative breast cancer (TNBC) treated with adjuvant chemotherapy. The prognostic effect is linear on the logarithm of the hazard of breast cancer events: with each 10% increment in TIL quantity, risk of invasive recurrences or death, risk of distant recurrences or death, and risk of death alone significantly decrease by 13% (95% CI 9% to 17%), 17% (95% CI 12% to 22%), and 16% (95% CI 11% to 21%), respectively, after adjusting for other prognostic clinicopathological factors.[11] These data, collected from 2148 patients, provide strong evidence to support TIL as the first biological prognostic biomarker in the TNBC subtype and significantly add to prognostic information provided by traditional staging by anatomical burden, with potential to upstage and downstage patients according to level of TIL. In our pooled analysis of individual patient

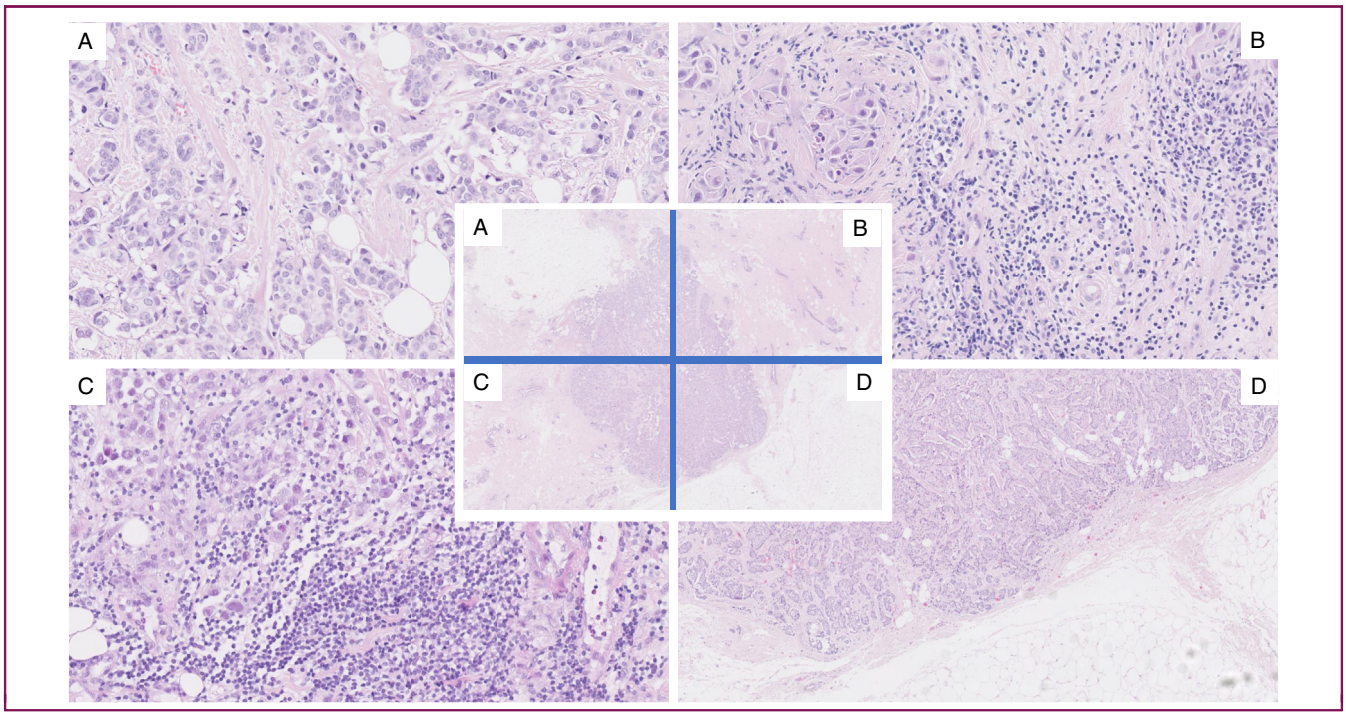

Figure 1. How to assess tumor-infiltrating lymphocytes (TILs) on a full-face hematoxylin and eosin-stained slide.
Panels A-D are regions of the full slide. The stromal score of TILs in zone A is 0%; in zone B it is 60%; in zone C it is 90% and in zone D it is 0%. The TIL score is averaged across each zone: 0% + 60% + 90% + 0% = 150%/4 = 37%, which is averaged by pathologists to 40%.

data, a full prognostic model integrating clinicopathological plus TILs was developed and validated and showed excellent calibration of predicted survival probabilities across studies.

The highest tertile in the node-negative population, corresponding to a cut-off of 30%, was proposed to divide patients into TIL high or low groups. When focusing on stage 1 patients with at least 30% TILs, 3-year estimates for invasive disease-free survival, distant disease-free survival, and overall survival (OS) were 93% (90% to 97%), 98% (95%-100%), and 99% (98%-100%), respectively. In 2019, Park et al[12] reported on the outcomes of patients with stage 1 TNBC from retrospective studies and one randomized trial, all of whom had not received any adjuvant systemic chemotherapy. Patients with high TIL (≥30%) had 5-year distant disease-free survival of 97% (93% to 100%), and OS of 98% (95% to 100%). These data support the inclusion of TIL % into any future clinical trials of new therapies in early-stage TNBC and may be of even higher relevance as we move into an era of checkpoint inhibitor incorporation. Interestingly, in young women, a higher cut-off may be needed.[13] Some high TIL patients with stage 1 TNBC therefore can have an excellent prognosis even without adjuvant chemotherapy.[12] This question likely cannot be answered without a prospective clinical trial but inclusion of TIL quantity in routine pathology reports would allow real-world data about prognosis for untreated patients with stage 1 TNBC in the future. According to our reproducibility studies, a value of 30% led to high pairwise concordance rates between pathologists across the ring studies (from 0.81 to 0.93) using both core biopsies and full-face sections.[3]

A common question is what is the value of a prognostic biomarker, as it does not directly change patient management, and the current standard treatment for nearly all patients with early-stage TNBC is cytotoxic chemotherapy? While this is currently true, it is foreseeable that this may change in the future in an era of immunotherapy. It is also notable that we routinely report on other prognostic markers that do not directly change management such as histological grade and lymphovascular invasion. Regardless, the TIL biomarker has now been included in several international guidelines for early-stage disease. This includes the 2019 St Gallen consensus conference for early-stage breast cancer[14]; the European Society of Medical Oncology (ESMO) Guidelines for early-stage breast cancer[15] as well as the World Health Organization (WHO) Blue Book on breast tumor classification.[16]

In early-stage HER2+ breast cancer, positive associations between high TIL and improved prognosis are observed in the presence of trastuzumab-containing chemotherapy regimens as well as in both early- and late-stage patients with trastuzumab and pertuzumab.[17,18] A meta-analysis is ongoing with the Oxford Early Breast Cancer Trials collaborative group (EBCTCG) of several phase III adjuvant trastuzumab studies and TIL evaluation has been performed in all studies. In the ShortHER study, for primary HER2+ patients with higher TIL (≥20%) a shorter duration of trastuzumab seemed adequate; by contrast, patients with lower TIL had good outcomes only with the full 1-year duration.[19] These data further support TIL levels as a good prognostic marker and may aid efforts in identifying patients suitable for investigating less cytotoxic chemotherapy or shorter-duration anti-HER2 therapy. Prognostic studies in dual

Table 1. Proposed TIL cut-offs for breast cancer subtypes, early versus late stage and with or without PD-1/PD-L1 inhibition

Breast cancer subtype	Chemotherapy	With or without PD-1/PD-L1 inhibition	Early or late stage	Proposed cut-off (study)
TNBC	AC/FEF/FAC ± taxane	Without	Early stage At diagnosis	30% identified as a satisfying cut point for node-negative patients [for N0 patients with TIL ≥30%, 3-year invasive disease-free survival was 92% (95% CI 89% to 98%), distant disease-free survival was 97% (95% CI 95% to 99%) and overall survival was 99% (95% CI 97% to 100%)] <40 years could be considered to be excluded at this cut point given the PARADIGM data.
HER2+	FEC+D/V ± trastuzumab AC-CMF versus AT-CMF	Without	Early stage At diagnosis	Higher TIL is clearly prognostic 20%-40% cut-off commonly used Possible interaction with the effect upon adding pertuzumab (APHINITY) Possible use in duration of anti-HER2 agents
TNBC after neoadjuvant chemotherapy Residual disease	AC/FEF/FAC ± taxane	Without	Early stage At residual disease after neoadjuvant chemotherapy	20% identified as a good cut point and could add prognostic information to yp stage as well as RCB Prognostic effect is linear. *Consider defining a cut-off with PD-L1 status*
HER2+	Docetaxel with trastuzumab and pertuzumab	Without	Late stage Primary or metastatic sample Metastatic sample preferred First-line setting	20% was prognostic Higher TIL was associated with long-term responders (CLEOPATRA)
TNBC	AC/EC-taxane	With	Early stage Neoadjuvant setting	<10% versus ≥10% was not predictive of increased magnitude of benefit from atezolizumab (associated with increased pathological complete response rate with and without atezolizumab) (GeparNuevo, IMpassion031) *Suggest considering lower levels such as <5% versus ≥5% in future studies with PD-L1 status*
TNBC	Nab-paclitaxel No chemotherapy	With	Late stage Primary or metastatic sample Metastatic sample preferred First line setting	≥10% identified those at increased magnitude of benefit from atezolizumab Combined with PD-L1 positivity and TIL ≥10%, progression-free survival and overall survival effect was very strong with atezolizumab (IMpassion130). ≥5% identified those that benefitted from pembrolizumab over chemotherapy (KEYNOTE119; KEYNOTE086) *Consider combination of TIL with PD-L1 status*
HER2+	Trastuzumab Trastuzumab-emtansine	With	Late stage Primary or metastatic sample Metastatic sample preferred Second or beyond	≥5% identified those at increased magnitude of benefit from PD-1/PD-L1 inhibition (KATE2, PANACEA) *Consider combination of TIL with PD-L1 status*

For detailed references, see Supplementary Table S1 and S2, available at https://doi.org/10.1016/j.annonc.2021.07.007.
For early-stage setting, continuous values may be useful in a prognostic model.
AC, doxorubicin/cyclophosphamide; CI, confidence interval; CMF, cyclophosphamide, methotrexate, 5FU; D/V, docetaxel or vinorelbine; EC, epirubicin, cyclophosphamide; FAC, 5FU, doxorubicin, cyclophosphamide; FEC, 5FU, epirubicin, cyclophosphamide; RCB, residual cancer burden; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

anti-HER2 targeting agents versus trastuzumab are also ongoing.

Unlike predictive markers where a therapeutic decision is binary (i.e. give or not give in the case of trastuzumab or endocrine therapy), a prognostic marker's role is more complex in guiding clinicians on therapeutic decisions based on risk of recurrence balanced against absolute benefits of the treatment and associated morbidity. In this way, prognostic tools that integrate all such factors may be most helpful. Useful examples of this are UK PREDICT and Adjuvant Online. We have also developed a prognostic tool calculator for patients with early-stage TNBC that incorporates anatomical stage as well as TIL measures on www.tilsinbreastcancer.org. A prognostic biomarker may also be of most value in the HER2+ subtype, whereas survival now is excellent for stage 1 patients with adjuvant trastuzumab and paclitaxel.[20] Hence the question of how to define who should receive dual anti-HER2 agents and the subgroups in which the newer and dual HER2 targeting agents should be evaluated is of high importance and will involve incorporation of prognostic markers.

Interestingly, TIL does not seem to have a strong prognostic significance in the advanced setting[21] in the context of trastuzumab or lapatinib alone.

Prognostic and predictive value of TIL in the era of checkpoint inhibition

Immune biomarkers such as TIL will likely have a role in the setting of checkpoint inhibition (Table 1, Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2021.07.007). In advanced breast cancer, it has been shown that enrichment for PD-L1 expression on tumoral immune cells seems to be a prerequisite for the benefit to atezolizumab and pembrolizumab in TNBC and HER2+ BC[22-26] (Figure 2).

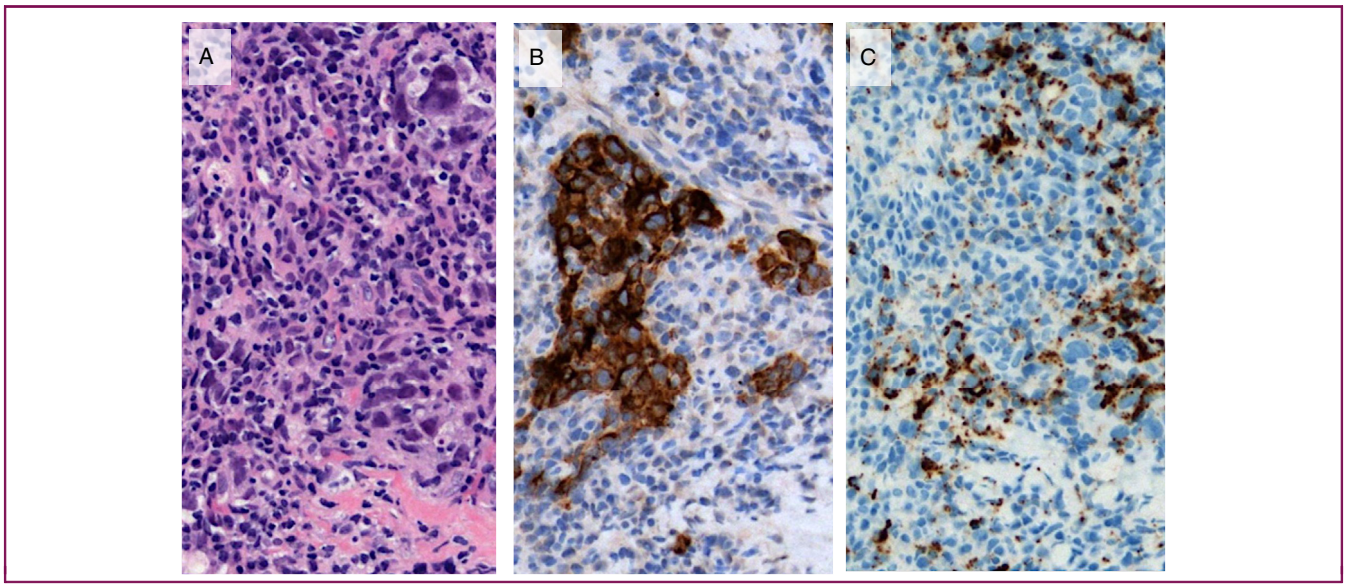

Figure 2. Heterogeneity of the tumor microenvironment in triple-negative breast cancer.
(A) In hematoxylin and eosin staining the tumor tissue consists of a mixture of tumor cells and immune cells. (B) Pan-Ck identifies the tumor cells; (C) PD-L1 staining (SP-142) with increased PD-L1 expression predominantly in the tumor-infiltrating immune cells. The images show consecutive section of the identical tissue area.

We have investigated the relationship between the TIL quantity and response to pembrolizumab in the setting of patients with advanced disease and a PD-L1 'Combined Positive Score' (CPS) $\geq$1 (PD-L1 IHC 22C3 pharm Dx assay). We found that in PD-L1+ patients, defined by a CPS score of $\geq$1%, TIL quantity of $\geq$5% further improved identification of those at higher chance of responding to pembrolizumab in two single-arm phase II studies involving patients with advanced TNBC and HER2+ BC who had had prior treatment.[21,22] Both markers were reported to have a moderate correlation (R = 0.45-0.55) suggesting that about 25% of variation in one marker is explained by the other, while also providing independent information.

In a randomized study (KATE2) evaluating the addition of atezolizumab to TDM-1, treatment effects in patients that were PD-L1+ using the VENTANA SP142 assay (41.5% of the intent-to-treat population) were estimated as follows: hazard ratio (HR) 0.60 (0.32-1.11) for progression-free survival and HR 0.55 (0.22-1.38) for OS. The VENTANA SP142 assay evaluates PD-L1 expression on the immune cells, with positivity defined as $\geq$1% staining. In a subgroup analysis of this study, higher TIL levels were also associated with PD-L1 positivity and increased benefit for both progression-free survival and OS in the TIL high group (i.e. TIL $\geq$5%). A similar effect was observed for those who were PD-L1+.[20]

The phase III KEYNOTE-119 study randomized patients who have had prior treatment for advanced TNBC to receive either chemotherapy of physician's choice or pembrolizumab as monotherapy. In this study, patients were enrolled regardless of their PD-L1 status. While this study did not show an OS benefit of pembrolizumab in the intent-to-treat population, in a *post hoc* analysis increasing benefit was observed in subset analyses of patients with higher CPS PD-L1 scores. Consistent with this, patients with TIL quantity $\geq$5% derived significant survival improvement from pembrolizumab compared with chemotherapy, but in those with low TIL levels (<5%) chemotherapy treatment resulted in better survival. These data provide the first evidence of the predictive ability of the TIL biomarker on its own in patients with advanced TNBC.[27]

In the first-line setting or in the context of untreated metastatic disease, the IMpassion130 phase III registration study evaluated the addition of atezolizumab to nab-paclitaxel, which resulted in the approval of atezolizumab for this breast cancer subtype if patients were positive for PD-L1 in the SP142 assay (40% of the intention-to-treat population; progression-free survival HR 0.62 (95% CI 0.49-0.78); OS HR 0.67 (95% CI 0.53-0.86)). In this landmark phase III study TIL, CD8, and PD-L1 IHC expression evaluated using the Ventana SP142 assay were moderately correlated and all immune markers could identify subgroups of women more likely to benefit from the addition of atezolizumab, with the PD-L1 SP142 assay seemingly appearing 'the best' in this dataset. While the median TIL cut point was 5%, $\geq$10% was used in a *post hoc* exploratory subgroup analysis.[28] Higher TIL was associated with a greater magnitude of benefit to the addition of atezolizumab, but most notable was the effect when combining both PD-L1+ and TIL high biomarkers: these 'immune-rich' tumors seemed to derive the greatest benefit.[29] We await analyses from the KEYNOTE-355 study[30] to help us understand if, in first-line patients with metastatic TNBC, the TIL cut point for predictive benefit is also useful at 10%, with and without the PD-L1 assay. The TIL biomarker is easy to read for all patients with advanced breast cancer and is independent from PD-L1 IHC staining. Hence TIL could be considered as part of routine reporting now in patients with advanced TNBC and HER2+ breast cancer, as it can provide helpful supporting information on the baseline immune status of the patient.

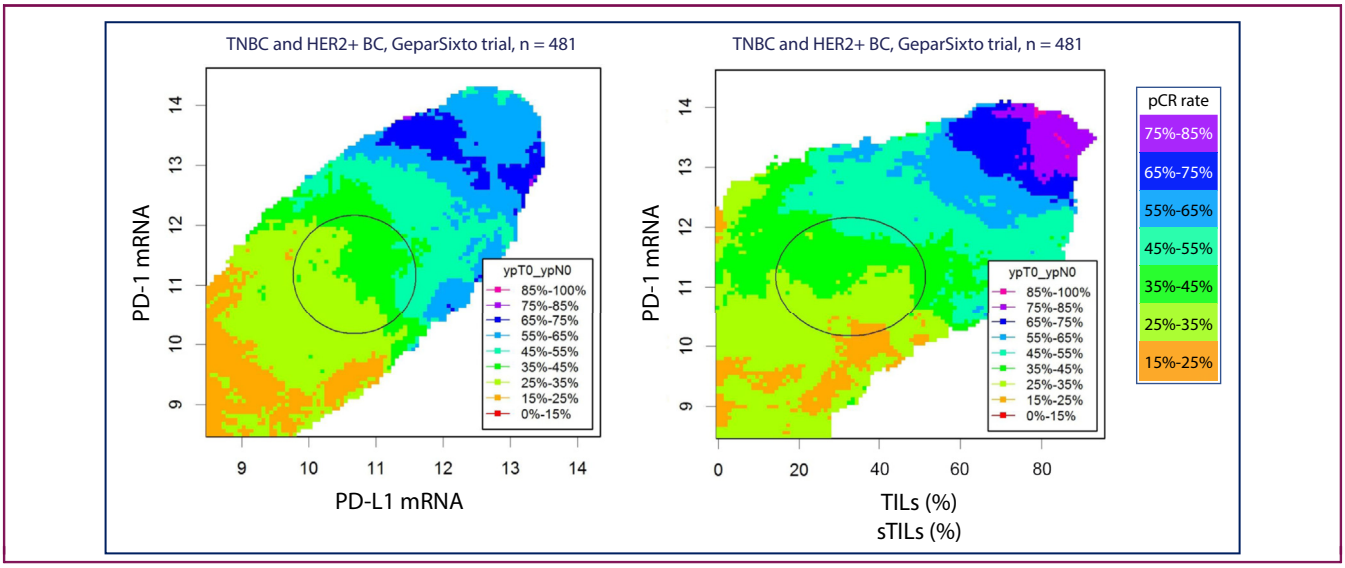

Figure 3. Chemotherapy-response maps: explorative analysis of combinations of immune markers in the GeparSixto trial (n = 481).
(A) Combination of two immune messenger RNAs (mRNAs; PD-1 and PD-L1). (B) Combination of PD-1 and tumor-infiltrating lymphocytes (TILs). The response rates of the different tumor groups are color coded, resulting in pathological complete response (pCR) rate between 15% and 85%. The figure shows that increased levels of PD-1 and PD-L1 are linked to increased pCR rates and that, in this setting, TILs can replace PD-L1. In addition, it is evident that immune activation is a continuous factor without biological cut points in the early-stage setting. TNBC, triple-negative breast cancer.

In the early-stage setting, evidence is still accumulating, but at present, unlike in advanced disease where TILs/PD-L1 appear to be predictive of benefit to PD-(L)1 inhibition, neoadjuvant treatment of primary TNBC with higher TILs/PD-L1 expression is associated with higher rates of pathological complete response rates (pCRs) independent of treatment, including chemotherapy with or without PD-(L)1 checkpoint inhibition.[31,32] Hence in the early setting, for those patients considered PD-L1—, we speculate that tumor-specific immunity can potentially be generated where none previously existed.[33] Lately, long-term outcomes have been reported as positive for the addition of PD-1—PD-L1 agents to standard chemotherapy.[34,35] As a result, as it is unclear how increases in pCR rates will convert to long-term survival outcomes when targeting PD-(L)1, thus making it difficult to interpret immune biomarker data using pCR endpoints.

In the neoadjuvant setting, exploratory analysis (Figure 3) also suggests that variables on immunity, including PD-L1, are best assessed in a continuous manner, as is the recommendation for TILs, as both help predict response in patients with TNBC and HER2+ disease. Because the substrate of PD-L1 evaluation, irrespective of the assay and assessment method used, is the immune cells in the tumor, it is clear that if there are no immune cells present, then the PD-L1 assay cannot be positive. Similarly, it is less likely that when a patient's tumor has high TILs, the PD-L1 stain will unlikely be negative. This supports the notion that TIL quantity should be evaluated together with the PD-L1 assays, in order to help interpret the staining or another assay may need to be used.[36]

An interesting observation is that 30%-50% of early TNBC with high TILs and high PD-L1 expression will have a pCR with classical chemotherapy—these immune biomarkers are positive prognostic factors. Therefore, clinical cohorts in the metastatic situation will be enriched for non-pCR tumors, implying that these patient cancers will have a different tumor-immune context.

TIL COMPOSITION AND POTENTIAL CONTRIBUTIONS OF OTHER BIOMARKERS

In primary breast tumors, TIL predominantly comprises T cells, and as the TIL quantity increases, so does the proportion of $CD8^+$ relative to $CD4^+$ T cells, indicating a cytotoxic T-cell response.[37] Can definition of specific T-cell subsets better define who benefits from T-cell checkpoint inhibition, apart from PD-L1? Investigations into the heterogeneity of $CD8^+$ T cells have revealed that additional markers such as *CD103 (ITGAE), CD39 (ENTPD1),* and *TCF1* may further discriminate a specific tumor-reactive T-cell population.[38] However, these seem to be only present on a subset of the T cells seen in the tumor, and there are a large proportion of 'bystander' T cells present in the tumor, likely attracted by the cytokine release but reactive against other antigens such as viruses.[39-41] It is also plausible that many T cells that are effective against the tumor epitopes are suppressed by the hostile microenvironment and/or by tumor intrinsic factors. More research in this area will be critical.

It is yet to be determined how other immune markers or gene signatures can add or refine TIL quantity or PD-L1 IHC status as a biomarker of response to PD-1/PD-L1 targeting agents. Incorporation of new markers will require similar technical validation as above, as well as accumulating extensive evidence on their added value (relative to cost, false negatives, and technical complexities). Defining the exact T or other immune cells that are the target of a

https://doi.org/10.1016/j.annonc.2021.07.007

durable response to T-cell checkpoint inhibition, remains, however a high priority for the field, independent of their biomarker potential.

The prohibitive cost of PD-L1 assays, especially in low- to-middle-income countries, could be mitigated by triaging patients using the sTILs on H&E slides to select cases for further analysis. Furthermore, while the PD-L1 field becomes increasingly complex in daily practice, with different PD-L1 assays across different regulatory environments, TILs can be assessed in a uniform manner using the main custom slide that is used by all pathologists worldwide, namely, a H&E-stained slide. Recently, representatives of the TILs Working Group, together with representatives of the College of American Pathologists, the European Society of Pathology, the Latin American Society of Pathology, and the European Working Group of Breast Screening Pathology have commented on how the current assay-approval policies, exemplified by the PDL1-assays, are leading to more, not less, complexity and confusion in daily practices. We propose concrete solutions to improve the current assay approval pathway.[42]

PD-L1 assays remain the approved companion diagnostic test for atezolizumab and pembrolizumab for patients with advanced TNBC, atezolizumab and that of pembrolizumab, anticipated to be approved in the near future. The controversies over PD-L1 quantification, from both a technical standpoint and reproducibility, and cut-off issues are well documented in other solid tumor types and apply also to breast cancer. We propose to integrate both measures[31]; however, the best way to incorporate these two markers will require both to be measured in ongoing immunotherapy trials.

Interestingly, we and others have observed TIL decreases with more lines of treatment.[43] It is very likely that tumor burden, representing multiple clones capable of evading the immune system as well as resistant to multiple lines of therapies, tempo of disease growth as well as marked local and systemic immunosuppression contribute to this observation. Hence, combinations of immune and antitumor therapies will likely be needed.

For those in whom a metastatic tumor biopsy is difficult or not feasible, peripheral blood markers are a promising area of response prediction and remain relatively easy to access. Peripheral levels of $CD8^+$ $PD\text{-}1^+$ T cells may provide a parameter of those most likely to respond to a PD-1 inhibitor. Along this theme, T-cell receptor (TCR) sequencing of peripheral blood may also prove useful: expansion of TCR clones in peripheral blood, particularly if one can prove that these are tumor derived (through TCR sequencing of tumor), has been shown to correlate with a host antitumor immune response in other cancer types.[44] Genomic correlates such as tumor mutation burden, high levels of homologous recombination deficiency or the presence of various homologous recombination deficiency mutations (such as BRCA) may also modify our choices for immunotherapy prescription in the near future, though much work needs to be done on the technical aspects of these biomarkers.[45]

Ultimately, these tests too may be cost prohibitive, and specific working groups will need to dedicate time to understand the relative advantages of simple versus complex assays, and cost-benefit in different clinical situations as the data emerge. It seems clear, however, that several measurements of the immune response are likely to provide clinically and biologically useful information about patients who respond to immune agents.

FUTURE DIRECTIONS

There is still much work to do in the field of biomarkers and immunotherapy for breast cancer, as well as for other cancers. The method of evaluating TIL in breast cancer can act as a framework for evaluating TIL quantity in other tumor types. In the future, machine learning techniques may help us further standardize TIL evaluation, as well as identifying important immune subsets. Finally, we advocate the incorporation of TIL quantity measurements as routine part of daily pathology for patients with breast cancer, both early and late stage. This is highly feasible as TIL reading can be easily added to routine pathology, especially in low-to-middle-income countries where the costs of PD-L1-assays could be prohibitive. We conclude that TIL quantity can provide highly relevant information as we go forward into a future that involves immunotherapy for our patients with breast cancer.

ACKNOWLEDGEMENTS

We thank Elizabeth Blackley and Jeannette Parrodi for their help with the table.

FUNDING

SL is supported by the National Breast Cancer Foundation of Australia Endowed Chair, the Breast Cancer Research Foundation, New York and the National Health and Medical Research Council, Australia. Roberto Salgado is supported by the Breast Cancer Research Foundation (BCRF, grant no. 17-194).

DISCLOSURE

SL receives research funding to her institution from Novartis, Bristol Meyers Squibb, Merck, Roche-Genentech, Puma Biotechnology, Pfizer, Eli Lilly, and Seattle Genetics; has acted as a consultant (not compensated) for Seattle Genetics, Pfizer, Novartis, BMS, Merck, AstraZeneca, and Roche-Genentech; has acted as a consultant (paid to her institution) for Aduro Biotech, Novartis, GlaxoSmithKline, and G1 Therapeutics. SA receives research funding to her institution from Novartis, Bristol Meyers Squibb, Merck, Roche-Genentech, Amgen, and Celgene; has acted as a consultant (not compensated) for BMS, Merck, and Roche-Genentech. RS reports nonfinancial support from Merck and Bristol Myers Squibb; research support from Merck, Puma Biotechnology, and Roche; and personal fees from Roche for an advisory board related to a trial-research project. CD reports personal fees from Novartis, personal fees and travel support from Roche, personal fees from

MSD Oncology and Daiichi Sankyo, grants from Myriad Genetics and German Breast Group (GBG), and other support from Sividon Diagnostics/Myriad, outside the submitted work. In addition, CD has patents EP18209672 and EP20150702464 pending, as well as a patent Software (VMscope digital pathology) pending. SM reports personal fees outside the submitted work for statistical advice from IDDI and Janssen Cilag, and for data and safety monitoring memberships from Hexal, Steba, IQVIA, Roche, Sensorion, Biophytis, Servier, and Yuhan. SLo reports grants and other from Novartis during the conduct of the study; grants and other from AbbVie, Amgen, Celgene, Novartis, Pfizer, Roche, Daiichi-Sankyo, and AstraZeneca; other from Seattle Genetics, PriME/Medscape, Lilly, Samsung, EirGenix, BMS, Puma, MSD, and Merck KG; personal fees from Chugai; grants from Immunomedics (all outside the submitted work). In addition, SiL has a patent EP14153692.0 pending. Other is for advisory role and/or presentations fees paid to institution unless otherwise stated. JB has declared no conflicts of interest.

REFERENCES

1. Sistrunk WE, MacCarty WC. Life expectancy following radical amputation for carcinoma of the breast: a clinical and pathologic study of 218 cases. *Ann Surg*. 1922;75(1):61-69.
2. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406(6797):747-752.
3. TILs Education: What They Are and What They Do. YouTube. Available at https://www.youtube.com/watch?v=aPa-pXIBBlU. Published January 5, 2021. Accessed February 1, 2021.
4. Kos Z, Roblin E, Kim RS, et al. Pitfalls in assessing stromal tumor infiltrating lymphocytes (sTILs) in breast cancer. *NPJ Breast Cancer*. 2020;6:17.
5. Elmore JG, Longton GM, Carney PA, et al. Diagnostic concordance among pathologists interpreting breast biopsy specimens. *J Am Med A*. 2015;313(11):1122-1132.
6. Gilchrist KW, Kalish L, Gould VE, et al. Interobserver reproducibility of histopathological features in stage II breast cancer. An ECOG study. *Breast Cancer Res Treat*. 1985;5(1):3-10.
7. Pruneri G, Lazzeroni M, Bagnardi V, et al. The prevalence and clinical relevance of tumor-infiltrating lymphocytes (TILs) in ductal carcinoma *in situ* of the breast. *Ann Oncol*. 2017;28(2):321-328.
8. van Bockstal M, Baldewijns M, Colpaert C, et al. Dichotomous histopathological assessment of ductal carcinoma *in situ* of the breast results in substantial interobserver concordance. *Histopathology*. 2018;73(6):923-932.
9. Hendry S, Salgado R, Gevaert T, et al. Assessing tumor infiltrating lymphocytes in solid tumors: a practical review for pathologists and proposal for a standardized method from the international immunooncology biomarkers working group: part 1: assessing the host immune response, TILs in invasive breast carcinoma and ductal carcinoma *in situ*, metastatic tumor deposits and areas for further research. *Adv Anat Pathol*. 2017;24(5):235-251.
10. Simon RM, Paik S, Haynes DF. Use of archived specimens in evaluation of prognostic and predictive biomarkers. *J Natl Cancer Inst*. 2009;101(21):1446-1452.
11. Loi S, Drubay D, Adams S, et al. Tumor-infiltrating lymphocytes and prognosis: a pooled individual patient analysis of early-stage triple-negative breast cancers. *J Clin Oncol*. 2019;37(7):559-569.
12. Park JH, Jonas SF, Bataillon G, et al. Prognostic value of tumor-infiltrating lymphocytes in patients with early-stage triple-negative breast cancers (TNBC) who did not receive adjuvant chemotherapy. *Ann Oncol*. 2019;30(12):1941-1949.
13. De Jong VMT, Wang Y, Opdam M, et al. Prognostic value of tumour infiltrating lymphocytes in young triple negative breast cancer patients who did not receive adjuvant systemic treatment; by the PARADIGM study group. *Ann Oncol*. 2020;31:S303-S339.
14. Burstein HJ, Curigliano G, Loibls S, et al. Estimating the benefits of therapy for early-stage breast cancer: the St. Gallen international consensus guidelines for the primary therapy of early breast cancer 2019. *Ann Oncol*. 2019;30(10):1541-1557.
15. Cardoso F, Kyriakides S, Ohno S, et al. Early breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2019;30(8):1194-1220.
16. Hoon Tan P, Ellis I, Allison K, et al. The 2019 WHO classification of tumours of the breast. *Histopathology*. 2020;77:181-185.
17. Krop IE, Paulson J, Campbell C, et al. Genomic correlates of response to adjuvant trastuzumab (H) and pertuzumab (P) in HER2+ breast cancer (BC): biomarker analysis of the APHINITY trial. *J Clin Oncol*. 2019;37(suppl 15):1012.
18. Luen SJ, Salgado R, Fox S, et al. Tumour-infiltrating lymphocytes in advanced HER2-positive breast cancer treated with pertuzumab or placebo in addition to trastuzumab and docetaxel: a retrospective analysis of the CLEOPATRA study. *Lancet Oncol*. 2017;18(1):52-62.
19. Dieci MV, Conte P, Bisagne G, et al. Association of tumor-infiltrating lymphocytes with distant disease-free survival in the ShortHER randomized adjuvant trial for patients with early HER2+ breast cancer. *Ann Oncol*. 2019;30(3):418-423.
20. Tolaney SM, Barry WT, Guo H, et al. Seven-year (yr) follow-up of adjuvant paclitaxel (T) and trastuzumab (H) (APT trial) for node-negative, HER2-positive breast cancer (BC). *J Clin Oncol*. 2017;35(suppl 15):511.
21. Liu S, Chen B, Burugu S, et al. Role of cytotoxic tumor-infiltrating lymphocytes in predicting outcomes in metastatic HER2-positive breast cancer: a secondary analysis of a randomized clinical trial. *JAMA Oncol*. 2017;3(11):e172085.
22. Schimd P, Rugo HS, Adams S, et al., Impassion130 Investigators. Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol*. 2020;21(1):44-59.
23. Emens LA, Esteva FJ, Beresford M, et al. Atezolizumab plus trastuzumab emtansine (T-DM1) versus T-DM1 plus placebo in previously treated, HER2-positive advanced breast cancer: results from KATE2, a randomised, double-blind, phase 2 trial. *Lancet Oncol*. 2020;21:1283-1295.
24. Loi S, Giobbie-Hurder A, Gombos A, et al. Pembrolizumab plus trastuzumab in trastuzumab-resistant, advanced, HER2-positive breast cancer (PANACEA): a single-arm, multicentre, phase 1b-2 trial. *Lancet Oncol*. 2019;20(3):371-382.
25. Adams S, Schmid P, Rugo HS, et al. Pembrolizumab monotherapy for previously treated metastatic triple-negative breast cancer: cohort a of the phase 2 KEYNOTE-086 study. *Ann Oncol*. 2019;30(3):397-404.
26. Adams S, Loi S, Toppmeyer D, et al. Pembrolizumab monotherapy for previously untreated, PD-L1-positive, metastatic triple-negative breast cancer: cohort B of the phase 2 KEYNOTE-086 study. *Ann Oncol*. 2019;30(3):405-411.
27. Loi S, Winer E, Lipatov O, et al. Relationship between tumor-infiltrating lymphocytes and outcomes in the KEYNOTE-119 study of pembrolizumab versus chemotherapy for previously treated metastatic triple-negative breast cancer. Poster session presented at *the 42nd Annual San Antonio Breast Cancer Symposium (Immunooncology Biomarkers and the Immune Landscape of Breast Cancer)*. December 10-14, 2019; San Antonio, Texas.
28. Emens LA, Loi S, Rugo HS, et al. Impassion130: Efficacy in immune biomarker subgroups from the global, randomized, double-blind, placebo-controlled phase III study of atezolizumab plus nab paclitaxel in patients with treatment-naive, locally advanced or metastatic triple negative breast cancer. Abstract presentation at the *41st Annual San Antonio Breast Cancer Symposium (General Session 1)*. December 4-8, 2018; San Antonio, Texas.

29. Emens LA, Cruz C, Eder JP, et al. Long-term clinical outcomes and biomarker analyses of atezolizumab therapy for patients with metastatic triple-negative breast cancer: a phase 1 study. *JAMA Oncol*. 2019;5(1):74-82.
30. Cortes J, Cescon DW, Rugo HS, et al. KEYNOTE-355: Randomized, double-blind, phase III study of pembrolizumab + chemotherapy versus placebo + chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer. *J Clin Oncol*. 2020;38(suppl 15):1000.
31. Schmid P, Cortes J, Pusztai L, et al. Pembrolizumab for early triple-negative breast cancer. *N Engl J Med*. 2020;382(9):810-821.
32. Loibl S, Untch M, Burchardi N, et al. A randomised 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. *Ann Oncol*. 2019;30(8):1279-1288.
33. Voorwerk L, Slagter M, Horlings HM, et al. Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial. *Nat Med*. 2019;25(6):920-928.
34. Merck Sharp & Dohme Corp. Merck Announces Phase 3 KEYNOTE-522 Trial Met Dual Primary Endpoint of Event-Free Survival (EFS) in Patients With High-Risk Early-Stage Triple-Negative Breast Cancer (TNBC) [press release] (2021 May 13) [cited 2021 Jun 10]. Available at https://www.merck.com/news/merck-announces-phase-3-keynote-522-trial-met-dual-primary-endpoint-of-event-free-survival-efs-in-patients-with-high-risk-early-stage-triple-negative-breast-cancer-tnbc/. Accessed July 29, 2021.
35. Loibl S, Marmé F, Martin M, et al. Palbociclib for residual high-risk invasive HR-positive and HER2-negative early breast cancer—the Penelope-B trial. *J Clin Oncol*. 2021;39(suppl 14):1518-1530.
36. Gonzalez-Ericsson PI, Stovgaard ES, Sua LF, et al. The path to a better biomarker: application of a risk management framework for the implementation of PD-L1 and TILs as immuno-oncology biomarkers in breast cancer clinical trials and daily practice. *J Pathol*. 2020;250(5):667-684.
37. Savas P, Virassamy B, Ye C, et al. Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. *Nat Med*. 2018;24(7):986-993.
38. Kurtulus S, Madi A, Escobar G, et al. Checkpoint blockade immunotherapy induces dynamic changes in PD-1– $CD8^+$ tumor-infiltrating T cells. *Immunity*. 2019;50(1):181-194.e6.
39. Duhen T, Duhen R, Montler R, et al. Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nat Commun*. 2018;9(1):2724.
40. Byrne A, Savas P, Sant S, et al. Tissue-resident memory T cells in breast cancer control and immunotherapy responses. *Nat Rev Clin Oncol*. 2020;17(6):341-348.
41. Simoni Y, Becht E, Fehlings M, et al. Bystander $CD8^+$ T cells are abundant and phenotypically distinct in human tumour infiltrates. *Nature*. 2018;557(7706):575-579.
42. Salgado R, Bellizzi AM, Rimm D, et al. How current assay approval policies are leading to unintended imprecision medicine. *Lancet Oncol*. 2020;21(11):1399-1401.
43. Savas P, Loi S. Expanding the role for immunotherapy in triple-negative breast cancer. *Cancer Cell*. 2020;37(5):623-624.
44. Formenti SC, Rudqvist NP, Golden E, et al. Radiotherapy induces responses of lung cancer to CTLA-4 blockade. *Nat Med*. 2018;24(12):1845-1851.
45. McGrail DJ, Pilié PG, Rashid NU, et al. High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types. *Ann Oncol*. 2021;32(5):661-672.