Tumor Infiltrating Lymphocytes in Breast Cancer: Implementation of a New Histopathological Biomarker

13

Giuseppe Floris, Glenn Broeckx, Asier Antoranz, Maxim De Schepper, Roberto Salgado, Christine Desmedt, Dieter J. E. Peeters, and Gert G. G. M. Van den Eynden

Abstract

In this chapter we describe a promising new histopathological biomarker in immuno-pathology/oncology: tumor infiltrating lymphocytes (TILs). The semiquantitative assessment of TILs in breast cancer (and other tumors) is a well-defined histopathological parameter of which the assessment can easily be integrated in the standard examination of biopsies and resection specimens by the (surgical) pathologist. Focusing on breast cancer, we first summarize available evidence on the prognostic and predictive value of TILs in DCIS, ER+/HER2-, triple-negative, and Her2-positive breast cancer. We also describe the correlation between TILs and other biomarkers, the most notorious among pathologists being programmed death-ligand 1 (PD-L1).

Secondly, we describe the efforts of the International Immuno-Oncology Biomarkers Working Group (www.tilsinbreastcancer.org) to standardize TIL assessment (leading to standardized international guidelines), create awareness, and educate pathologists and oncologists. Finally, we briefly introduce new concepts and techniques that will in the coming years be introduced to further characterize the immune microenvironment in tumors and the interaction between tumor cells and inflammatory cells, such as the use of spatial single cell technologies and artificial intelligence. We believe these techniques should be integrated with the use of TILs and other biomarkers in clinical practice, for the benefit of our patients.

G. Floris
Department of Imaging and Pathology, KU Leuven, Translational Cell and Tissue Research Unit, Leuven, Belgium

Department of Pathology, University Hospitals Leuven, Leuven, Belgium
e-mail: giuseppe.floris@uzleuven.be

G. Broeckx
Department of Histopathology, Antwerp University Hospital, Edegem, Belgium
e-mail: glenn.broeckx@uza.be

A. Antoranz
Department of Imaging and Pathology, KU Leuven, Translational Cell and Tissue Research Unit, Leuven, Belgium
e-mail: asier.antoranzmartinez@kuleuven.be

M. De Schepper
Department of Pathology, University Hospitals Leuven, Leuven, Belgium

Department of Oncology, Laboratory for Translational Breast Cancer Research, KU Leuven, Leuven, Belgium
e-mail: maxim.deschepper@uzleuven.be

R. Salgado · G. G. G. M. Van den Eynden (✉)
Laboratory of Pathology and Cytology PA2, GZA/ZNA Hospitals, Antwerp, Belgium
e-mail: roberto@salgado.be

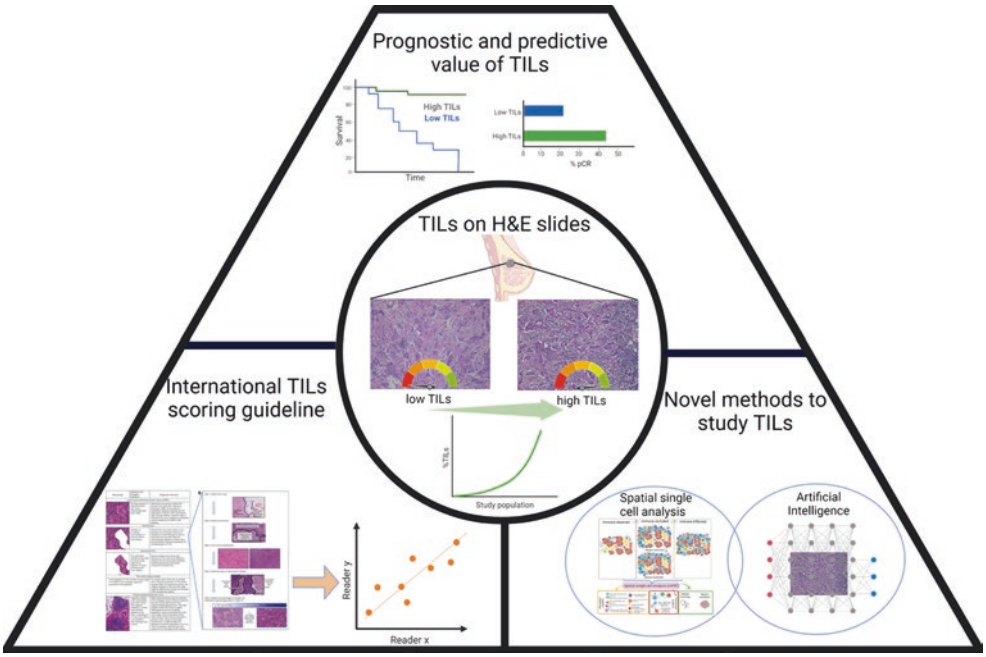
C. Desmedt
Department of Oncology, Laboratory for Translational Breast Cancer Research, KU Leuven, Leuven, Belgium
e-mail: christine.desmedt@uzleuven.be

D. J. E. Peeters
Histopathology, Imaging and Quantification Unit, CellCarta, Antwerp, Belgium

Laboratory of Pathology, AZ Sint-Maarten, Mechelen, Belgium

Human Molecular Genetics, University of Antwerp, Edegem, Belgium

L. A. Akslen, R. S. Watnick (eds.), *Biomarkers of the Tumor Microenvironment*, https://doi.org/10.1007/978-3-030-98950-7_13



Key Content Graphic. Figure created with BioRender.com

Take Home Lessons

- Tumor infiltrating lymphocytes (TILs) is a continuous marker showing strong positive correlation with survival outcomes especially in breast cancer patients with TNBC and HER2+ phenotypes, reaching level Ib of evidence for these groups of patients. The value of TILs in ER+/HER2- is currently discussed and poorly understood.
- The predictive value of TILs has been extensively investigated, being predictive for pathologic complete response in breast cancer patients treated with neoadjuvant chemotherapy. More recently, the introduction of immune checkpoint blocking therapy has shown promising positive interactions between TILs levels, PD-L1 expression, and response to therapy, strongly suggesting TILs as a potential predictive marker also in this context.
- Standardized international guidelines for the assessment of TILs on routine H&E stained slides are available, showing high grade of concordance among pathologists.
- A learning tool is freely available at www.tilsinbreastcancer.org to teach and assist in the scoring activities in the scientific community.
- The use of spatial single cell technologies and artificial intelligence will be the next step to understand the underpinning mechanisms of immune editing and unravel the interactions between tumor cells and the tumor immune microenvironment.

Part 1: Introduction

Over the last decade, our understanding of the interactions between cancer cells and the different components of the immune system has increased exponentially. Furthermore, we are developing strategies and medications to exploit this complex interplay for treating patients with cancer. The rapidly increasing number of possible targets and therapeutic options also makes it necessary to develop biomarkers that enable selection of the right therapy for the right patients. One of the reasons being that these innovative agents are extremely expensive.

The purpose of this chapter is to describe a very promising histopathological biomarker that can easily be incorporated in the standard assessment by (surgical) pathologists, namely tumor infiltrating lymphocytes (TILs). As further described, TILs can be assessed on a standard hematoxylin and eosin (H&E) stained slide, no special staining or additional test is needed. Although the same assessment method can be used for TILs in almost every tumor type and data show that its usefulness is not limited to breast cancer (BC) at all, the prognostic and predictive value has been most extensively demonstrated in BC. Therefore, this chapter will mainly focus on the use of TILs in the latter.

First, we will give an overview on the available evidence of the prognostic and predictive value of TILs in BC. We will furthermore give an overview of the guidelines as developed by the International Immuno-Oncology Biomarker Working Group on Breast Cancer and on the efforts and initiatives to standardize, teach, and distribute TILs assessment among

pathologists. The purpose of these international, academic efforts is to harmonize and streamline TILs assessment and to avoid the origination of an unworkable chaos as we have seen happening, for instance, in the field of PD-L1 assessment, with many different assays and assessment methodologies. In the last section we will shortly elaborate on future evolutions in TILs assessment.

Part 2: Evidence on Prognostic and Predictive Value of TILs

Evidence of the Prognostic and Predictive Use of TILs in Breast Cancer

Depending on the expression at the protein level of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER-2), and Ki-67, BC patients are subdivided into clinically and biologically meaningful surrogate intrinsic molecular categories. Treatment decisions are largely dependent on the combinations of these biomarkers, which show strong predictive value concerning anti-hormonal therapy in ER-positive tumors and anti-HER2 therapy in HER-2 positive tumors. A subset of tumors lacking expression ER, PgR, and HER2 (i.e., triple-negative breast cancers, TNBC) are associated with a poor prognosis and are mostly treated with standard chemotherapy [1, 2]. Interestingly, a growing body of evidence suggests that also in BC the amount of TILs relate to a better prognosis and are potentially predictive for response to chemotherapy [3]. The association with higher TILs and better prognosis in breast pathology was first proposed long time ago in a series investigating pathological features linked to improved prognosis after radical mastectomy [4]. Also, the paradox of the so-called medullary carcinoma of the breast which is a poorly differentiated BC (usually TNBC) associated with high level of peritumoral inflammation and indolent clinical course provided further evidence for this observation [5]. This is in line with the concept that a tumor mass is composed not only by tumor cells but also by non-neoplastic cells forming the so-called tumor microenvironment (TME). Stromal cells in the tumor microenvironment establish intimate interactions with the cancer cells, functioning as an external layer of modulation in cancer progression [6, 7]. Inflammatory cells play a major role in this process, as postulated by the theory of the cancer immunoediting [8]. Three phases, namely elimination, equilibrium, and escape seem to modulate the interactions between cancer cells and immune system. Although not yet fully understood, this process seems to be strongly regulated by the underlying genetic alterations in cancer cells and their capacity to escape or stimulate the immune system [8–10]. Overall, in cancer, high levels of immune infiltrates are a pre-requisite for response to novel therapeutic strategies like immune checkpoint blocking immunotherapy (ICB) and are considered to be a surrogate for high level of antigenicity (i.e., the presence of "foreign" epitopes in the cancer cells to be recognized by the immune system) [11, 12]. However, only a small number of cancers actually display a positive relation between tumor mutational burden (TMB), neoantigens, and CD8+ TILs (melanoma, endometrial cancer, non-small cell lung cancer, colorectal cancer). Breast cancer generally exhibits relatively lower TMB and neoantigens, with no clear correlation with CD8+ T-cell infiltration [13]. This apparent paradox will be further discussed in the section about novel methods to assess TILs.

The International Immuno-Oncology Biomarkers Working Group (see further; also referred to as TILs-WG) has recently issued guidelines to help pathologists in scoring the mononuclear immune infiltrate with a standardized scoring method, based on regular H&E staining [14–16]. The strength of this method resides in its prompt implementation, which can be easily translated into daily clinical practice. Like BC tumor grading which arguably constitutes the most important prognostic tool in breast pathology, the TILs scoring method is based on evaluation of H&E stained slides, making this tool accessible to all pathology laboratories around the world, and it is promising to become a novel standard of practice in breast pathology [17, 18].

TILs are not normally distributed across the BC molecular subtypes and in addition the clinical context may add a further layer of heterogeneity. In older series, about 17% of BC showed dense TILs infiltration within the tumor border irrespective of age, and high TILs were independently positively associated with a better survival only in patients younger than 40, implying strong differences in the interaction between BC and immune system according to tumor biology [19]. Specific associations with mutations or copy number alterations may also influence their prognostic impact [20–23]. A systematic review of 15 studies including almost 14.000 patients provided a global picture of the heterogeneous distribution of TILs across BC molecular subtypes. The median percentage of BC patient showing no evidence of TILs is 16%, while 11% show high level of TILs. The comparison between the specific molecular BC subtypes indicated that a higher level of TILs infiltration is generally observed in TNBC and HER2+ tumors which show a median value of high TILs level in 20% and 16% of the patients, respectively. On the contrary, patients with ER+ tumors showed about three times less frequently high TILs level as compared to TNBC and HER2+. These results were also reflected by the amount of CD8+ T-cells, suggesting a different activation of the adaptive immune system depending on the molecular BC subtype [24]. A pooled analysis of six randomized clinical trials in the neoadjuvant setting performed by the German Breast Cancer Group confirmed in over 3000 patients that TNBC and HER2+ tumors have the highest

incidence of TILs as compared to ER+ BC [25]. High TILs levels were defined according to the definition of lymphocytic predominant breast cancer (LPBC), which is a terminology adapted from the hematopathological literature. LPBC refers from a pragmatic point of view to BC for which the relative percentage of mononuclear inflammatory infiltrate in tumor nests or stroma "outnumbers" the tumor cells, the cut-off values most frequently used are 50% or 60% of TILs [26, 27]. Currently no cut-offs are recommended by the TILs-WG, and the TILs scoring should be considered rather as a continuous variable [28]. Nevertheless, efforts made by the TILs group around specific cut-points show excellent level of concordance among pathologists (intraclass correlation coefficient [ICC] ≥0.7), being higher than those reported for established methods like the Nottingham grading system [16, 29].

Prognostic Significance of TILs in Ductal Carcinoma In Situ (DCIS)

Ductal carcinoma in situ (DCIS) is a segmental disease of the terminal duct-lobular units and represents a non-obligate precursor of invasive BC which is historically related to higher risk of developing invasive carcinoma as compared to normal population. DCIS is extremely heterogeneous and treatment options involve usually either breast conserving surgery with or without radiotherapy or radical mastectomy. However, the natural history of DCIS remains poorly understood, posing the question of patient overtreatment and better selection for treatment [30]. For this reason, the search for reliable prognostic markers of recurrence in patients with DCIS has enormously flourished in the past years, including research on TILs. A scoring method to assess the density of immune infiltrates in the periductal stroma has been proposed as well by the TILs-WG [28]. Cases of pure DCIS tend to have less dense immune infiltrate as compared to DCIS associated with micro-invasive focus or compared to invasive BC [31]. Higher nuclear grades, solid growth pattern, and comedo-necrosis are generally associated with higher TILs levels [32]. Comparably to invasive BC, the quantity and the quality of the immune infiltrate in DCIS seems to be related to the protein expression of ER, PR, and HER2. Agahozo et al. described high TILs levels in over 60% of HER2+ and TN DCIS, while ER+ DCIS showed remarkably lower levels of TILs with ER+/HER2+ DCIS showing intermediate levels [33]. Analysis of the qualitative composition of the TILs (e.g., higher levels of FOXP3+ cells, increased number of PD-L1+ cells, and lower levels of granzyme B) suggests that mechanisms of immune editing implicated in immune escape may be involved in the development of invasive BC, occurring as recurrence after DCIS or in invasive BC associated with DCIS [33–35]. However, the DCIS phenotype does not significantly influence the composition of the immune infiltrate [33]. DCIS carrying *TP53* mutations and higher levels of aneuploidy seems also to be related to higher TILs levels [34, 36]. Interestingly the proportion of DCIS cases with high TILs varies depending on the scoring method used [33, 37]. This discrepancy might be explained by the location and the density of immune infiltrates which may be extremely variable around ducts affected by DCIS. Therefore, the identification of the portion of periductal stroma that need to be considered for the scoring might be particularly challenging and have a strong impact on the results and their interpretation. Benchmarking of seven different scoring methods for TILs in DCIS has identified that the count of TILs touching the basement membrane of the ducts is the most reproducible and reliable method of all (so-called touching TILs) [38]. However, efforts made by different groups have revealed that scoring histopathological features in DCIS, including TILs, results in fair to moderate interobserver agreement requiring further refinement in the definition and education of pathologists [39–41]. These observations may explain the discrepancy in results reported by diverse independent groups in recent years, when the prognostic value of periductal TILs density was interrogated as prognostic marker for (ipsilateral) breast recurrence. Indeed, at present it remains unclear whether the density of DCIS-associated periductal TILs can predict recurrence [34, 38, 42–45] or not [36, 37, 40, 46] calling for a profound revision and harmonization of scoring methods to be used. Unraveling the interactions existing between DCIS and its periductal inflammatory microenvironment will be important to understand mechanisms of BC immune escape that may contribute to the selection of patients for ICB therapies [35].

Prognostic Significance of TILs in ER+/HER2- Breast Cancer in the Adjuvant Setting

ER+/HER2- BC largely corresponds to the IHC surrogate of the luminal molecular BC intrinsic subtype, as defined by transcriptome analysis. Depending on the proliferation markers, ER+/HER2- BC is subdivided into low proliferative (also referred to as luminal A-like) or high proliferative BC (also referred to as luminal B-like) [47]. Tumor grade, tumor size, and lymph-node status are traditionally used in the adjuvant setting to guide systemic therapy decisions in BC patients with luminal disease [48]. The recent clinical validation of the use of multigene signatures (MGS) has further refined this decision-making process, especially for post-menopausal patients [49–51]. Interestingly, high levels of TILs are supposed to influence MGS results when they are associated with BC that would be allocated to the luminal A-like category by standard histopathology [52]. This observation is perhaps the result of a bias introduced by the higher

proliferation level of the TILs, which may influence MGS results in these cases [53]. Data describing how TILs values influences MGS results in patients with luminal disease are limited and conflicting. In one study studying TILs densities in a retrospective monocentric cohort including 344 early BC patients (of which 187 ER+) treated with surgery and adjuvant systemic treatment, TILs level was not prognostic for overall survival and disease-free survival in ER+ tumors despite a negative correlation between TILs value and recurrence scores measured by the MGS Oncotype Dx [54]. On the contrary, another small retrospective monocentric study focusing on luminal tumors in the adjuvant setting showed a weak but statistically significant correlation between continuous TILs values and continuous recurrence scores values measured by Oncotype Dx [55]. However multivariable analysis demonstrated that Oncotype Dx scores was not an independent factor able to predict high TILs in this study, suggesting that tumor immune microenvironment may contribute to prognosis as well in luminal tumors [55]. Similarly, the retrospective-prospective translational analysis of the West German Study PlanB trial found a strong and significant correlation between TILs levels and recurrence scores as measured by Oncotype Dx. A model based on hormone receptor status, Ki67 and TILs levels was found to be predictive of Oncotype Dx results with an area under the curve of 0.80. The authors concluded that the impact of the TILs on prognosis might not necessarily be dependent on the association with proliferation [56]. Interestingly, previous in silico analysis performed on 15 publicly available databases illustrated that the interaction between inflammatory metagenes and prognosis in ER+ tumors may have a dual effect depending on the molecular subtype and proliferation status. In their study, Nagalla et al. found that 23% of highly proliferative ER+ tumors displayed high expression levels of inflammatory metagenes and were associated with excellent distant metastasis-free survival while an inverse relationship was observed in luminal B tumors with low proliferative activity [57]. Indeed, mitotic stress is considered a strong regulator of the immune surveillance in cancer cells [58, 59].

In this regard the integration of BC transcriptome and genome analysis seems to provide a novel twist to this complex background in ER+ tumors. Smid and collaborators found that the upregulation of TIL signatures and better prognosis may be triggered only by specific patterns of somatic mutations (also referred to as mutational signatures) independently from the cell cycle [60]. In their study the mutational signatures 3 and 13 were particularly associated to higher TILs signatures and better prognosis. A recent study performed on TNBC patients with or without germline *BRCA* mutations (which possibly correspond to signature 3) seems to confirm this observation. Indeed, the number of patients with TILs >10% was significantly higher in the patients carrying the mutation as opposed to the wild-type group, although the TILs infiltrates were globally similar between the two groups [61]. Conversely, a high number of non-synonymous mutations are generally linked to high proliferation, high tumor grade, and poor prognosis [60, 62]. However, specific mutational signatures or other genetic mutations that may trigger TILs (e.g., microsatellite instability) may be rarely encountered in BC with luminal biology (e.g., 86% of ER+ BC did not show the mutational signature 3 in the report from Smid et al.) [60, 63]. Importantly, TILs scores measured on tumor tissue are strongly correlated to the expression level of immune signatures in transcriptome analyses and represent a reliable surrogate of the immune response within a tumor [64, 65]. It remains to be ascertained whether the presence of high level of TILs on histology is a reliable predictor for a specific mutational signature in ER+ tumors and if the LPBC category is a clinically meaningful category in these tumors, despite its inconsistent definition.

Considering the previous observations, it is not surprising that the literature reports conflicting results when it comes to study the prognostic value of TILs in ER+ tumors in the adjuvant setting. Indeed, in a recent systematic review, the percentage of non-LPBC ER+ BC cases was estimated to be almost 95% [24]. Many authors failed in finding significant associations between TILs densities evaluated on H&E stained slides and prognosis in different clinical trials, however no particular distinction was made between luminal subtypes [26, 66, 67]. Others confirmed the lack of prognostic information of TILs in the global population of ER+ BC patients, but in subgroup analysis found a positive prognostic association in the high proliferative tumors (although in [68] this was only borderline) and an inverse prognostic correlation in the low proliferative tumors [68, 69]. In addition, a recent meta-analysis has revealed that LPBC category in ER+ tumors displays worse overall survival but has no effect on disease-free survival in the 4 high quality studies included. However, the authors acknowledge that results should be taken with caution considering the limited number of studies available [70]. Interestingly, special BC types that are usually associated to a luminal phenotype like invasive lobular and micropapillary carcinomas show generally low TILs densities and inverse prognostic correlation [23, 71–73]. On the contrary, male BC, which is characterized by ER expression in about 99% of the cases and displays lower incidences of BC with special histology, shows a positive correlation with prognosis in cases with high TILs levels [74, 75]. The reduced presence of CD8+ T-cells in the epithelial compartment of luminal invasive BC has been advocated as possible cause for the adverse prognosis observed in some studies [76, 77].

Given the highly heterogeneous outcomes observed in the ER-positive disease, it is conceivable that subgrouping breast carcinomas by ER expression alone can be insufficient to describe clinically meaningful associations between outcomes and immune response. So far clinical utility and

validity of TILs assessment in ER+/HER2- tumors has been inconsistent and warrants further studies before standard application in the clinic [78].

Prognostic Significance of TILs in HER2+ Breast Cancer in the Adjuvant Setting

HER2+ BC is classically defined by its peculiar strong membranous HER2 expression which is readily visible by IHC on low magnification under the microscope [79]. The strong HER2 protein expression is generally the consequence of the *HER2* gene amplification, which renders HER2+ BC cells addicted to the activation of its metabolic cascade [80]. The major evidence for this oncogenic addition is provided by the dramatic improvement of survival outcomes that have been achieved thanks to the introduction of the targeted anti-HER2 therapy in combination with cytotoxic chemotherapy as standard of care [81, 82]. Nevertheless, HER2+ BC remains very heterogeneous disease that can be driven by different underlying molecular mechanisms depending in first instance on the ER+ or ER- status [83]. Additionally, as illustrated by transcriptomic analysis with the PAM50 MGS classifier, both ER+/HER2+ and ER-/HER2+ IHC BC subgroups can be further divided into four major molecular BC subtypes, although with different proportions. This observation has led to the introduction of a new classification within HER2+ tumors, resulting in the category of the HER2-enriched tumors, which also may benefit from anti-HER2 therapy despite lacking strong HER2 protein expression, for which a different distribution of TILs has been observed depending on HER2 IHC score levels [84, 85]. Based on these observations, one may argue that mechanisms of immune editing may have a different impact on prognostic and predictive outcomes in HER+ BC. For instance, ER+/HER2+ BC generally displays less TILs as compared to ER-/HER2+ BC [66, 86]. Moreover, the ER status seems also to influence survival outcomes and response to treatment in HER2+ BC. However, transcriptomic analysis suggests that when dealing with HER2-enriched tumors, the ER status may lose its relationship with clinical outcomes [83]. In this regard, the study of the expression of T-cell metagenes in BC molecular subtypes indicated a strong positive prognostic correlation between high expression level of the lymphocyte-specific kinase (LCK) metagene and HER2-enriched tumors, irrespective of the ER status [87].

The BIG 02-98 trial as well as the FinHER trial failed to find a positive prognostic association between high TILs level and disease-free or overall survival [26, 66]. On the contrary, Dieci and collaborators found that both intratumoral TILs (iTILs) and stromal TILs (sTILs) were associated with improved OS in two multicentric phase III trials in the adjuvant setting. For each 10% sTILs increase, an 18% reduction in death risk was observed for HER2+ patients (adjusted HR 0.82, 85% CI 0.69–0.96, $p = 0.02$) [67]. In a selection of 945 out of 3505 HER2+ BC patients enrolled in the phase III N9831 adjuvant study (<50% of the entire cohort), sTILs infiltrates were investigated to study recurrence-free survival in relation to type of systemic treatment (i.e., chemotherapy alone *vs* chemotherapy + trastuzumab). The predefined cut-point at 60% was used to define LPBC on whole slide sections. Of the 945 cases analyzed 94 (± 10%) were LPBC, the majority of the LPBC showed ER+/HER2+ phenotype and displayed less recurrence events as compared to non-LPBC ($n = 8$ vs 154, respectively) [88]. Similarly to what was observed in the BIG 02-98, patients with high TILs level in the N9831 study had improved recurrence-free survival in the chemotherapy arm [26, 88]. However, as opposed to the FinHER study in the N9831, the benefit of adding trastuzumab to the backbone of systemic chemotherapy was not observed; the statistical power to exclude or confirm ER influence was too limited [66, 88]. A retrospective analysis of the NRG/NSABP-B31, a large phase III trial assessing the benefit of trastuzumab in combination with chemotherapy in BC patients with HER2+ disease in the adjuvant setting, revealed that higher TILs values measured on H&E (both semicontinuous and categorical, LPBC = 50%) were significantly associated with improved disease-free survival but were not predictive of trastuzumab benefit. Of note, whole slide sections of about 82% of the patient population enrolled in the trial ($n = 1581/1931$) were analyzed, 100 of which were additionally reviewed by 6 pathologists of the TILs-WG achieving 90.8% concordance between the main reviewer and the 6 additional observers (mean value). Interestingly, high TILs scores showed also strong correlation with groups of patients showing high benefit from anti-HER2 targeted therapy as defined by an 8-gene prediction signature on transcriptome analysis [89]. Intriguingly, the same predictive signature has been recently validated in the B-31 and N9831 study by an independent validation, suggesting that specific immune markers may capture different biological outcomes as opposed to global TILs assessment by H&E [89, 90] Biomarker analysis performed in about 70% ($n = 866/1253$) of the samples collected in the negative non-inferiority phase III ShortHER trial (i.e., 9 weeks vs 52 weeks adjuvant Trastuzumab) showed an improved five-year rate distant disease-free survival for patients with ≥20% sTILs as compared to patients with <20% sTILs (95.7% vs 91.1%; $p = 0.025$), and it was found that the distant disease-free survival rate was excellent for both treatment arms in patients with higher TILs value suggesting TILs guided de-escalation options for HER2+ BC patients [91]. *PIK3CA* mutations were associated with higher sTILs and provided a favorable five-year disease-free survival in the HER2-enriched tumors as established by PAM50 MGS [20]. More

recently TILs have been included in a multivariable prognostic tool that has the potential to inform treatment choices in BC patients with HER2+ disease [92].

Altogether the evidence collected in the adjuvant setting suggests a strong positive prognostic value for high level of TILs in HER2+ BC. Two recent meta-analyses confirmed these results [70, 93]. HER2-enriched tumors are supposed to retain higher TILs level as compared to luminal molecular subtypes assessed by PAM50.

Somatic activating mutations in the *HER2* are rare events encountered in not more than 1–3% of BC causing HER2 activation in an alternative way as compared to BC with *HER2* gene amplification. BC carrying *HER2* mutations are not mutually exclusive with *HER2* gene amplification, correlate more frequently with lobular histology, and are supposed to be a mechanism of endocrine resistance and for anti-HER2 therapy as well [84, 94]. Recent clinic-pathologic review of primary tumors of metastatic BC patients carrying *HER2* mutations revealed low sTILs in nine out of 13 patients and LPBC in one out of 13 patients (median sTILs = 5%; mean sTILs = 15%; LPBC defined as ≥50% sTILs); the distribution across the surrogate molecular subtypes was overall comparable to that observed in the general BC population [95].

Prognostic Significance of TILs in TNBC in the Adjuvant Setting

Almost one fifth of all BC lacks ER, PR, and HER2 expression and is referred to as TNBC. This group of BC is generally characterized by aggressive biological behavior, poor prognosis, earlier age at presentation, and higher risk of metastasis. Although the TNBC definition is clinically meaningful and reflects the lack of specific therapeutic options, this terminology is from the biological point of view highly inaccurate [96]. Therefore, it was not surprising that at the transcriptomic level TNBC could be subdivided into at least four different intrinsic molecular subtypes, including the immunomodulatory or the basal-like immune activated which display a favorable prognosis [97, 98]. Bridging the TNBC gene expression profiles to the clinic represents the main challenge in the coming years (e.g., tumors showing high AR expression by IHC may be considered a surrogate for Luminal Androgen Receptor (LAR) subtype) [99]. Interestingly, as already suggested by Lehmann and colleagues, the immunomodulatory subtype shared transcriptomic features with those described in medullary carcinomas of the breast, which are classically characterized by TNBC status, high inflammatory infiltrates, and good prognosis despite their high grade on histology [5, 100]. TILs assessment on H&E is thus potentially the best surrogate marker available to date to recapitulate the immune-related molecular subtype within the TNBC group, which may be not necessarily seen as distinct entity [25, 101]. What is thus the evidence accumulated so far in TNBC using the TILs scoring method proposed by the TILs-WG?

In the retrospective analysis of the BIG 02-98 about 70% (2009/2887 patients enrolled in the study) of the tumor tissue was retrieved to measure iTILs, sTILs as continuous variable and LPBC as categorical variable with cut-point at 50%; outcomes were overall survival and disease-free survival. Receptor status was unknown in a bit more than 400 patients, leaving 256 patients in the TNBC category. In this subgroup of patients 10% increment of TILs as continuous variable in the stromal compartment was associated with a statistically significant improved disease-free and overall survival with 15% and 17% reduction in risk, respectively. Categorical TILs for TNBC with the LPBC phenotype showed 92% five-year disease-free and overall survival rates, which were comparable to that observed in the luminal subgroup where the TILs had no prognostic significance. Of note, interactions with the type of systemic chemotherapy (anthracycline only *vs* anthracycline-docetaxel) were observed only in the group of BC patients with HER2+ disease [26]. In the FinHER study (n = 134 TNBC patients) no association with overall survival was observed probably due to the low number of events [66]. In another retrospective analysis of two large phase III trials in the adjuvant setting including 199 TNBC patients, similar results were observed. The ten-year overall survival rate was 89% and 68% for high TILs and low TILs, respectively (HR 0.44, 95% CI 0.18–1.10, p = 0.07), no interaction with anthracycline-based chemotherapy was found [67]. Two independent groups at both sides of the Atlantic Ocean validated further the strong prognostic value of TILs in TNBC. Adams and colleagues measured TILs on whole tissue slides stained by H&E in a selection of 481 out of 506 tumor blocks of TNBC patients enrolled in two phase III studies sponsored by the Eastern Cooperative Group (ECOG E2197 and E1199) [102]. Pruneri and coworkers analyzed TILs scores in whole slide H&E-stained sections from 647 tumors collected in the context of the adjuvant phase III trial from the International Breast Cancer Study Group Trial 22-00 [103]. Both studies showed in multivariable analysis a remarkable similarity in hazard ratio values when sTILs were considered as continuous variable to assess the risk reduction in disease-free survival (HR = 0.90 [95% CI 0.82–0.97;p = 0.01] in Pruneri et al., HR = 0.86 [95% CI 0.76–0.98; p = 0.02] in Adams et al., respectively), distant recurrence-free interval (HR = 0.83 [95% CI 0.74–0.94; p = 0.004] in Pruneri et al., HR = 0.81 [95% CI 0.69–0.95; p = 0.01] in Adams et al., respectively), and overall survival (HR = 0.83 [95% CI 0.74–0.93; p = 0.001] in Pruneri et al., HR = 0.82 [95% CI, 0.68–0.99; p = 0.04] in Adams et al., respectively), confirming the strong prognostic value of TILs in TNBC patients. The LPBC category showed similar results as well [102, 103]. In 2019 a pooled analysis of pro-

spective-retrospective data collected from over 2100 TNBC patients enrolled in 9 large studies performed in the adjuvant setting was published and confirmed the strong prognostic value of the TILs scoring method on H&E, finally reaching level Ib evidence [104]. In this study the average sTILs value was 23% (mean 15%, quartile range 10–30%), lower TILs quantities were significantly associated with older age, larger tumors, more lymph-node involvement, and tumors with lower grade. This pooled analysis confirmed a very strong, statistically significant linear correlation between increment in sTILs quantity and better invasive disease-free, distant disease-free, and overall survival with hazard ratio in the range of 0.83 and 0.86, being very similar to what was observed in the original studies. A test for heterogeneity indicated no or minimal heterogeneity among the studies; iTILs showed similar results. Interactions with the type of adjuvant systemic chemotherapy (anthracycline alone *versus* anthracycline-taxane) were found to be not statistically significant. Further analysis was performed to test the performance of a predefined cut-point in predicting prognosis. To this extent the higher quartile of the sTILs values across the entire cohort was chosen (30%) and tested for the three endpoints. The cut point at 30% remained statistically significant for all outcomes and it contributed significant improved prognostic value in all nodal categories. Further statistical analysis was performed to demonstrate the additional independent prognostic value of TILs quantities as compared to standard clinic-pathological parameters; however, this was true only for the stromal component of the TILs. Based on these results a clinicopathological prognostic model combining classical clinic-pathological features with sTILs values has been made freely available on the website of the TILs-WG: www.tilsinbreastcancer.org [104].

The Role of TILs in the Neoadjuvant Setting, in the Metastatic Setting and in Immune Checkpoint Blocking Immunotherapy

Neoadjuvant Setting

The achievement of pCR provides improved overall survival and better event-free survival but is not validated as surrogate endpoint yet [105]. For BC patients not achieving pCR, the measurement of residual disease remaining in the tumor bed after neoadjuvant treatment entails as well important prognostic information which is better captured by standardized scoring methods [106–108]. Therefore, the identification of reliable biomarkers able to predict pCR may have important implications for the prognosis of BC, especially for those carrying luminal B-like, HER2+, and TNBC tumors and may inform further treatment approaches in post-neoadjuvant phase [105, 109, 110]. The current TILs scoring method has been applied for the first time by Denkert et al. as a predictive tool to predict pathologic complete response (pCR) in patients enrolled in two neoadjuvant anthracycline/taxane-based studies (GeparDuo, n = 218, training cohort; and GeparTrio, n = 840, validation cohort) in a total of 1058 diagnostic core needle biopsies [27]. In this seminal paper the investigators demonstrated that the percentage of sTILs and iTILs was significantly and independently associated with increased pCR values. When exploring the so-called LPBC phenotype (defined as 60% TILs), the authors observed a significant improvement with pCR rates of 41.7% and 40% in the GeparDuo and GeparTrio, respectively. They concluded that TILs scores were able to identify a subpopulation of patients showing improved response to neoadjuvant chemotherapy and observed that LPBC displayed different histomorphological features as compared to the criteria of medullary carcinoma [27]. Perhaps also for this reason the medullary carcinoma is no longer considered a special subtype of BC in the latest edition of the World Health Organization Classification of breast tumors [18]. The addition of carboplatin to the anthracycline/taxane backbone in the neoadjuvant scheme showed an increase in pCR rates up to 59.9% in LPBC suggesting for the first time a strong interaction between TILs and the type of treatment when carboplatin was added (the odds of pCR increased 3.71-fold in LPBC tumors compared with a 1.01-fold increase in non-LPBCs) [64]. This finding, however, was not further validated in another trial from the same group performed at a later point in time [111]. TILs were found to be a strong and independent predictor of pCR especially in TNBC and HER2+ tumors [27, 64, 111, 112]. Independent confirmation of the predictive value of TILs on the diagnostic pretherapeutic core biopsy is also available from the literature.

In the NeoALTTO study 455 women with HER2+ early breast cancer were randomly assigned to 1 of 3 neoadjuvant treatment arms: trastuzumab, lapatinib, or the combination for 6 weeks followed by the addition of weekly paclitaxel for 12 weeks, followed by 3 cycles of fluorouracil, epirubicin, and cyclophosphamide after surgery. Retrospective review on H&E of 85% (n = 387/455) of the diagnostic biopsies collected in this study revealed a non-linear correlation between TILs level and pCR rates, with an odds ratio of 2.6 [(95%CI, 1.26–5.39; p = 0.01] when TILs levels were greater than 5%. Importantly, patients with sTILs levels greater than 40% achieved excellent 3-year event-free survival rates regardless of pCR status, being equal to those observed in patients achieving pCR, confirming the strong prognostic value of the TILs [86]. The positive correlation between TILs quantity and pCR was also observed in a multi-institutional study. In this study experienced breast pathologists across Europe tested the reproducibility of the TILs scoring method on pretreatment biopsies of TNBC patients treated with neoadjuvant chemotherapy (anthracycline- taxane+/− carboplatin). TILs scores done on the same set of slides in two consecutive circulations (initially by 16 and then subsequently by 19 participants) demonstrated on multivariable analysis that

increasing TILs levels (both as continuous variable and with 10% increment) were predictive for higher pCR rates [113]. Moreover, albeit modestly, the interobserver concordance was improved in the second circulation possibly pointing to a beneficial effect of the training, as suggested by the TILs-WG [113, 114]. Asano and colleagues reviewed retrospectively 177 cases of early BC patients treated in their institution with neoadjuvant chemotherapy and found positive association with increased pCR and improved survival endpoints in TNBC and HER2+ tumors but not in the luminal ones [115]. In the LAR-like subtype TILs values as categorical variable measured on the pre-treatment core biopsy (≥30%) were predictive for pCR but association with AR expression levels on IHC lacked statistical significance. Interestingly, the multiple correspondence analysis described in one of the clusters a possible association of high AR expression with low TILs, older age, obesity, and residual disease after therapy, possibly indicating a different response to therapy in relation to AR status, body weight, and TILs [116]. Recently, a large retrospective analysis performed by Hamy et al. described the association between sTILs quantity on pretherapeutic core needle biopsies and pCR by reviewing over 700 institutional cases encompassing all surrogate molecular subtypes. The authors found that pCR was positively associated with higher levels of TILs only in patients with TNBC disease and described a non-linear association. Similarly, the levels of TILs in the pretherapeutic biopsy showed a non-linear improvement of disease-free survival only in the whole population and in the TNBC patients. This effect was additionally related to the type of therapy used, since a statistically significant positive interaction was observed only in patients who received other treatment than anthracyclines +/− taxane or taxane alone (HR = 0.968; 95% CI, 0.944–0.994; $p = 0.014$). No significant associations were observed in the luminal-like and HER2+ subtypes concerning TILs scores in the pre-treatment biopsy [117]. On the contrary, the pooled analysis performed by the German Breast Cancer Group in 3771 pre-treatment biopsies collected from patients treated in six neoadjuvant clinical trials showed a significant association between improved pCR rates and increased TILs quantities, regardless of surrogate molecular subtype. In the whole population the pCR rates were indeed significantly higher in the tumors with high TILs level as compared to those observed in the intermediate or low TILs groups ($High^{\geq 60\%TILs}$ = 44% vs $Intermediate^{11-59\%TILs}$ = 27% vs $Low^{\leq 10\%TILs}$ = 22%; $p < 0.0001$). The magnitude of pCR rates was the highest in the TNBC category, nevertheless the effect of TILs in diverse clinicopathological categories seemed to be the same in all molecular subtype according to a post-hoc univariate logistic regression analysis. When prognostic outcomes were investigated in the 2570 patients of which complete follow-up data were available, the scientists found that disease-free and overall survival were differentially affected by molecular subtype. In univariate analysis high TILs predicted a longer disease-free survival in TNBC and HER2+ tumors, a longer overall survival only in the TNBC subgroup, and a reverse association with overall survival in the luminal category. In the multivariate analysis, the inclusion of baseline features and pCR rates showed that high TILs were no longer associated with better outcomes in TNBC and HER2+ tumor, while the inverse association with overall survival was retained in the luminal tumors. Kaplan-Meier analysis showed similar results. Interestingly, a post-hoc analysis of the survival performed on the surrogate molecular subtypes showed for both endpoints a mixed effect in the luminal category. For instance, high TILs were associated with a shorter disease-free survival in the grade 1–2 tumors (HR = 1.132; 95% CI = 1.04–1.233; $p = 0.004$) as opposed to grade 3 tumors, where high TILs predicted longer recurrence outcome (HR = 0.879; 95%CI = 0.787–0.981; $p = 0.021$) possibly suggesting once again a dual effect of TILs based on proliferation [25, 57, 69]. Specific analysis on ER status in the HER2+ tumors showed no statistically significant difference on both outcomes in the study of Denkert and colleagues [25]. A recent meta-analysis confirms the results from Denkert et al. concerning pCR rates but suggested a publication bias for the results observed in the luminal tumors [70]. While another meta-analysis specifically focused on TNBC studies confirmed, after review of 37 studies, the predictive value of high TILs for achieving higher pCR rates and improved survival endpoints [118].

In the past years, the variation in quantities of sTILs in response to chemotherapy has gained interest, because of the reported retained prognostic value of the TILs in relation to the partial response after neoadjuvant chemotherapy. In particular, the combination of the residual cancer burden (RCB) score and class with TILs may provide additional prognostic information that might be useful to inform clinicians for further therapies [109, 119] . The interest in this type of approach stemmed from initial reports based on small series, in which increasing densities of TILs were observed in relation to increasing level of response to neoadjuvant paclitaxel therapy in core biopsy-resection specimen matched samples and correlated with the level of apoptotic activity [120]. The evidence so far collected is greater for TNBC as compared to the other molecular subtype/surrogate. Dieci et al. performed a retrospective multi-institutional study, where sTILs and iTILs were assessed on a representative H&E slide from the resection specimen post-neoadjuvant chemotherapy ($n = 278$). Chemotherapy regimens were based on anthracycline +/− taxane schemes, and further adjuvant chemotherapy was given to 32% of the patients. High TILs levels were associated with tumor size smaller than 20 mm and negative lymph node status. In multivariate analysis, 10% increment in sTILs were associated with a statistically significant reduction in risk of metastasis (HR = 0.86; 95% CI = 0.77–0.96; $p = 0.01$) and reduction in risk of death (HR = 0.86;

95% CI = 0.77–0.97; $p = 0.01$). High TILs in residual disease predicted a longer metastasis-free and overall survival 5-year rates as compared to low TILs levels (81.5% vs 46% and 91% vs 55%, respectively). This difference remained significant only in the category of patients with positive lymph nodes +/− ypT2 tumors for the metastasis-free survival endpoint ($p = 0.005$) [121]. However, no correlations with RCB scores or classes were made. Patients with high TILs in residual disease showed an increase of TILs in the post-neoadjuvant resection specimen as compared to diagnostic biopsy ($n = 19$) [121]. This contrasted with what is reported by Loi and colleagues in which the matched pre- and post-neoadjuvant specimens showed a reduction in TILs levels associated with residual disease ($n = 39/111$; $p = 0.07$). No meaningful associations with survival endpoints were observed based on the TILs changes occurred during neoadjuvant treatment. Nevertheless, TILs in the residual disease post-neoadjuvant treatment did show a linear and statistically significant association with improved relapse free and overall survival also after correction for confounders in the multivariate analysis [122]. In another retrospective analysis Luen et al. matched sTILs pre-treatment on core biopsy with sTILs values post-treatment in 375 BC patients with TNBC disease. pCR cases were excluded from analysis. About 50% of the patients showed either increase or decrease in sTILs after neoadjuvant treatment, globally resulting on average in a non-statistically significant reduction of −3% in the post-treatment specimen. sTILs were statistically significantly inversely correlated with the ypTN and with the stage, however no correlation was observed with RCB class. Survival analysis showed that high sTILs in the residual disease were significantly associated with longer relapse free and overall survival only in patients with RCB class II, but not in the RCB class III. These results indicate that TILs can provide independent and additional prognostic information in patients with RCB class II [123]. Hamy and colleagues provided description of the pre- and post-neoadjuvant dynamics in a more comprehensive cohort of patients involving over 700 cases encompassing all molecular subtypes. TILs level in the residual disease indicated a dismal prognosis only in the HER2+ tumors, while it had no impact on prognosis in TNBC or luminal tumors. Additionally, they also demonstrated a reduction in TILs level following neoadjuvant chemotherapy across the whole study population (mean pre-treatment TILs = 24.1% vs mean post-treatment TILs = 13%; $p = <0.001$); this difference remained significant also in all molecular subtypes, but the magnitude was higher in HER2+ and TNBC samples. In addition, they found an inverse correlation between TILs level variations and TILs level pre-therapy, while a decrease in TILs was strongly associated with pCR [117]. Similar results have been reported on studies based on computational pathology in a series of over 500 cases, in which the increase of TILs density post-neoadjuvant therapy was negatively associated with the likelihood of pCR [124, 125].

The value of TILs scores as a predictive marker of response in chemotherapy-free schemes of neoadjuvant treatment is less established in the literature and to some extent controversial. Dieci et al. reported on the added value of baseline high TILs scores and high Ki-67 labeling index in predicting response, in a hypothesis generating study in which 77 patients treated with neoadjuvant aromatase inhibitors were enrolled. In this study non-ductal histology was found to be predictor of poor response [126]. A subsequent analysis done on the same cohort of patients showed that high TILs were significantly associated with non-luminal subtypes (26%) with basal-like showing the highest levels when tumors were classified according to the PAM50 MGS classifier. CIBERSORT analysis provided evidence for a more pro-inflammatory background in these tumors, suggesting that these tumors may be better candidate for other treatment strategies [127]. Reduced PR expression in the post-therapy specimens has been found to be related to lack of inflammatory infiltrate post-neoadjuvant endocrine treatment according to a retrospective monocentric study of 132 patients [128]. The dynamics of TILs and relationship with neoadjuvant endocrine therapy was studied in 119 patients treated with neoadjuvant letrozole for 4 months prior to curative intended surgery as part of a clinical phase II study conducted by the Danish Breast Cancer Group (DBCG). Diverse histopathological methods for response were applied and correlated with pre- and post-therapy TILs shifts. The investigators recorded globally a 6% increase in TILs values after neoadjuvant letrozole. Nevertheless, ductal histology and reduction of TILs after therapy were predictive for pCR (regardless of type of evaluation method used) [129]. The increase in TILs was thus associated with poor response being consistent with results coming from transcriptomic analysis indicating resistance to letrozole in ER+ tumors with higher lymphocytic inflammation [130] However, in the CARMINA study the response to aromatase inhibitors was observed following an increase of TILs post-treatment [131]. The increase in CD8+ T-cells and in the ratio CD8+/Treg has been proposed as marker for response in patients treated with endocrine treatment in the neoadjuvant setting [132]. In the HER2+ tumors, novel chemo-free neoadjuvant approaches have been recently tested in BC patients with HER2+ tumors. In these clinical trials, the molecular HER2-enriched subtype as defined by the PAM50 MGS classifier was a strong predictor of response [133]. In addition, TILs have been also studied in this context providing further knowledge about the interaction of the tumor-associated immune environment in response to anti-HER2 therapies in HER2+ tumors. In the neoadjuvant PAMELA trial, 151 HER2 + BC patients have been treated with trastuzumab and lapatinib (+/− hormone treatment depending on ER status) for 18 weeks [133]. This trial pro-

vides valuable insights in the dynamic changes of TILs during treatment with targeted anti HER2 therapy, because it provides the comparison between the core biopsy collected at baseline *versus* the one collected at day 15 despite evaluations at the moment of surgery are missing. As already observed in other studies, TILs levels were statistically significantly differently distributed across molecular subtypes as defined by the PAM50 classifier. sTILs at baseline were found to be significantly associated with pCR only in univariable analysis. Combined anti-HER2 therapy induced a significant increase in TILs in most patients at day 15. These induced sTILs were found independently associated with pCR in multivariate analysis. Tumor cellularity was also evaluated at the two points in time and found to be positively associated with pCR in multivariate analysis. A combined score called CelTIL was then derived from both variables and was found to be a good predictor (both categorical and continuous) at day 15 for pCR [134]. The CelTIL score have been recently validated as categorical variable in the NeoALTTO trial, showing better 5-year event-free and overall survival rates in the high CelTIL tumors as compared to the low CelTIL tumors (EFS = 76.4%$^{\text{CelTIL-HIGH}}$ vs 59.7%$^{\text{CelTIL-LOW}}$; OS = 86.4% $^{\text{CelTIL-HIGH}}$ vs 73.5% $^{\text{CelTIL-LOW}}$) [135].

Finally, important patient related conditions may also influence the interactions between tumor cells and its surrounding microenvironment. Obesity is becoming a novel pandemic in the western countries, which is strongly associated with cardio-vascular conditions and cancer resulting in poor survival outcomes. Recently, Desmedt et al. have demonstrated that lipophilic cytotoxic drugs may result in reduced disease-free and overall survival in relation to increasing value of body mass index (BMI), which is probably related to the high distribution volume of these drugs in patients with obesity [136]. Based on these observations, a recent study reviewed 445 diagnostic biopsies collected from TNBC patients treated with neoadjuvant chemotherapy in two large tertiary centers, to study the relations between TILs and BMI [137]. High TILs were statistically significantly associated with pCR in lean patients but not in overweighted patients. The association between TILs and BMI was linear after formal statistical testing and showed a statistically significant interaction between TILs and BMI. A model showing the odds ratio of having pCR was built in function of TILs and BMI. This model clearly showed that the likelihood of having pCR in lean patients with high TILs was higher than that of overweighted patients with high TILs. Survival analysis showed better event-free and overall survival rates only in lean patients but not in overweighted BC patients (HR = 0.22[95% CI = 0.08 to 0.62; $p = 0.004$] vs 0.53 [95% CI = 0.26 to 1.08; $p = 0.08$] and HR = 0.22 [95% CI = 0.07 to 0.70; $p = 0.01$] vs HR = 0.65, 95% CI = 0.31 to 1.35; $p = 0.25$), although in multivariate analysis the interaction term was not statistically significant [137]. These results indicate qualitative differences in the composition of the inflammatory tumor microenvironment according to BMI which considering recent findings may be potentially informative for hypothesis generating trials using ICB therapy specifically in overweighted BC patients [138].

Metastatic Setting

The knowledge about immune and tumor interactions is quite limited and fragmented in the BC metastatic setting. One of the major hurdles in this field of research is tissue procurement, which prevents researchers in most of the cases from comparing matched samples. Indeed, metastasis can be anatomically difficult to reach, sample processing may impair the quality of the material or the tissue available is very limited. Nevertheless, testing metastasis for receptor status is now regarded a mainstay because it may better inform clinicians about therapeutic options and unravel molecular mechanisms of resistance and progression [139]. Given the strong prognostic information showed by the TILs in the early setting it is anticipated that also in the metastatic setting will be retained, providing useful insights in the understanding of the process of immune escape [140].

In general, the tumor microenvironment in metastasis shows lower densities of lymphocytic aggregates as compared to primary tumors, suggesting upregulation of mechanisms of immune evasion by the tumor cells [141–145]. BC metastasis in the lung displays higher TILs level as compared to other metastatic sites, while brain, skin, and liver show reduced TILs quantities [146]. Interestingly, liver metastases show different histologic growth patterns within the liver, each associated with prognosis in BC patients with oligometastatic disease [147, 148]. It remains to be ascertained how TILs relate to these histologic patterns and whether TILs can provide further prognostic information. BC patients with brain metastasis, for instance, show improved overall survival in the presence of immune infiltrate, gliosis, or hemorrhages, while necrosis is associated with shorter overall survival [149]. Additionally, transcriptomic analysis performed on matched samples demonstrated a significant downregulation of molecules that are associated with immune activation and upregulation of immune suppressive molecules in metastatic specimens in comparison to primary tumors, supporting the hypothesis of immune evasion in BC metastatic sites [150]. Survival analysis performed in a retrospective study in which metastatic lesions collected from 94 TNBC and HER2+ BC patients were evaluated to study TILs revealed a significant association between TILs and prognosis in both TNBC and HER2+ tumors [151]. Higher TILs in TNBC metastatic patients showed longer overall survival rates, while in HER2+ BC patients an inverse relation was observed [151]. In the Cleopatra study, on the contrary, after review of over 670 samples collected from HER2+ BC patients with metastatic disease, a significant positive

association between high TILs and prolonged overall survival was observed. Of note, in this study only a minority of samples were collected from metastatic sites ($n = 58$), and in less than 30 cases, it was possible to make a matched comparison with the primary tumors [152]. Two important considerations can be made based on these findings. First the survival analysis of the Cleopatra study most likely informs more about the interactions existing between the primary tumor and TILs rather than about those observed between metastatic site and TILs. Secondly, the biomarker analysis of this large clinical trials reflects the difficulties encountered in the tissue procurement in the metastatic setting. A possible solution to this problem may come from the establishment of post-mortem tissue donation programs that may provide useful information and unravel the underlying mechanisms of resistance, progression, and immune escape in metastatic BC patients [153, 154]. Several programs of this kind are currently running in no more than 15 locations around the world, including one recently initiated at our institution (University Hospitals Leuven; clinical trial number NCT04531696).

Immune Checkpoint Blocking Immunotherapy

High immune infiltrates together with high antigenicity are considered a pre-requisite for response to ICB therapies. Nevertheless, there seems to be a dissociation between TILs and formation of neoantigens in BC [13]. To date, the assessment of the program death-ligand receptor 1 (PD-L1) by IHC constitutes the only reliable predictive marker of response to ICB therapies. Two companion diagnostic antibodies have been validated and approved in the context of randomized clinical trial to select patients who may potentially respond to ICB therapies. The Ventana PD-L1 SP142 (Medical System Inc., Tucson, AZ, USA) and the PDL1 22C3 pharmDx (Dako North America, Inc., Carpinteria, CA, USA) are currently indicated as the companion diagnostics for selecting advanced TNBC patients for atezolizumab (anti PD-L1 antibody) and pembrolizumab (anti PD-1 antibody), respectively. Additionally, two different scoring methods should be applied to assess expression in BC specimens [155]. Table 13.1 provides a summary of the different scoring methods to be applied according to ICB therapy.

Figure 13.1 illustrates the PD1-PD-L1 interaction and mechanism of action of anti PD1/PD-L1 ICB therapy.

For the SP142 PD-L1 assay (Ventana, AZ, USA), the immune cell (IC) area score with cut-off at 1% has been validated in the context of the phase III randomized controlled trial for unresectable locally advanced or metastatic TNBC (IMpassion130: atezolizumab/placebo + first-line chemotherapy with nab-paclitaxel). A statistically significant longer progression free survival was observed in the intended to treat population as well as in the PD-L1+ population. The overall survival was not improved in the intended to treat population. Despite not formally tested due to the design of the study, an improved overall survival was observed in the PD-L1+ population treated with atezolizumab [157, 158]. The use of the PD-L1 IHC 22C3 pharmDx assay (Agilent, CA, USA) has been validated in the KEYNOTE-355 trial comparing physician's best choice systemic treatment (i.e., diverse cytotoxic agents) in combination with either placebo or pembrolizumab for the first-line treatment of inoperable locally recurrent or metastatic TNBC. The combined positive score (CPS) was used to identify patient populations that may respond to pembrolizumab. The CPS measures PD-L1 expression not only in immune cells (lymphocytes and macrophages) but also on tumor cells. The trial showed a significantly longer progression free survival for patients treated with the combination of chemotherapy+ pembrolizumab as compared to chemotherapy alone and CPS $\geq$ 10 [159]. However, the use of PD-L1 IHC as predictive marker remains controversial given the results obtained in another phase III trial, IMpassion131, which compared first-line paclitaxel with either placebo or atezolizumab in a similar population of patients with unresectable locally advanced or metastatic TNBC. In this trial, which showed negative results, the PD-L1+ status as measured by the SP142 assay did not show effect on outcomes end points [160]. Several factors may be responsible for these results, including treatment choice or the fact that PD-L1 assessment may be a poor predictive

Table 13.1 General overview of PD-L1 assays used in clinical trials and (for validated indications) in clinical practice, their companion staining platforms, associated scoring methods applied in clinical trials and companion anti-PD1/anti-PD-L1 monoclonal antibody drugs across tumor indications. In bold are indicated the drugs and respective companion diagnostic assays and scoring algorithms validated in clinical trials for patients with TNBC

Clone	Manufacturer/Platform	Scoring method	Companion drug
22c3	Agilent Dako/Dako Autostainer Link 48	TPS (%) = (#TC^{+ve} /total #TC) × 100	**Pembrolizumab**
28-8		**CPS = (#TC^{+ve} + #$IC_{Ly/M\varphi}{}^{+ve}$ /total #TC) × 100**	Nivolumab
73-10		TPS (%) = (#TC^{+ve} /total #TC) × 100	Avelumab
SP263	Ventana/Benchmark Ultra	$IC_{proportion}$ (%) = (#$IC_{mononuclear}{}^{+ve}$ /total #IC) × 100 IC_{area} (%) = (area occupied by $IC_{anytype}{}^{+ve}$/total tumor area) × 100	Durvalumab Avelumab
SP142		**IC_{area} (%) = (area occupied by $IC_{anytype}{}^{+ve}$/total tumor area) × 100** TPS (%) = (#TC^{+ve} /total #TC) × 100	**Atezolizumab**

TPS tumor proportion score, *CPS* combined positive score, *IC* immune cells, *TC* tumor cells, *Ly* lymphocyte, *Mφ* macrophage, # number/cell count

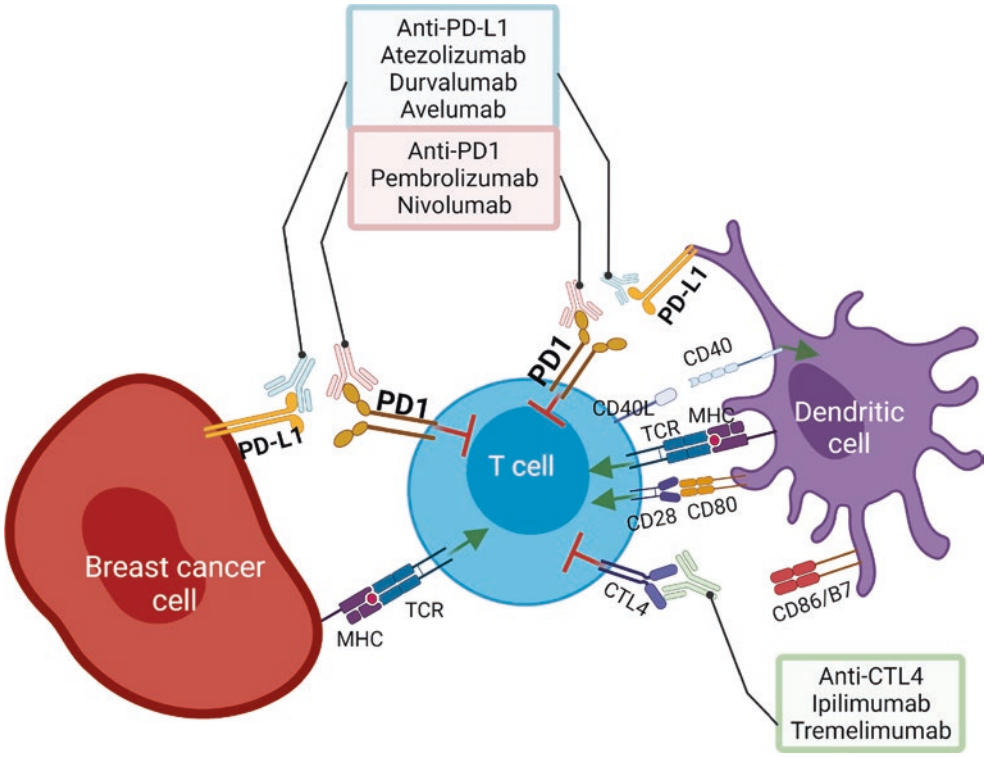


Fig. 13.1 Immune checkpoint localization in tumor cells, T cells, and antigen presenting cells. The figure illustrates the interactions between T cells, dendritic cells, and breast cancer cells. The interaction between check-point molecules results either in stimulatory signals (green arrow) or inhibitory signals (red). In the text boxes are shown the sites of action of the major monoclonal antibodies directed against immune checkpoint molecules, tested in different types of cancer. Treatment with one of the monoclonal immune checkpoint antibody blockers is supposed to block the inhibitory signals preventing activation of T cells and cytotoxic elimination of cancer cells. Figure created with BioRender.com. Adapted from [156]

marker of response [161]. Remarkably, also in the neoadjuvant setting of the phase III KEYNOTE-522 trial, where the addition of pembrolizumab to neoadjuvant anthracycline–taxane–carboplatin-based chemotherapy was studied, pCR rates were significantly improved in the combination treatment arm regardless of PD-L1 status as determined with the 22C3 pharmDx assay assessed with CPS at a cut-off of 1 or greater [162]. Similar results were observed in the phase III Impassion031 trial investigating the addition of atezolizumab to nab-paclitaxel and anthracycline-based chemotherapy in the neoadjuvant treatment of patients with early stage TNBC, suggesting a potentially different role for PD-L1 assessment in TNBC in the primary as opposed to the metastatic disease setting [163]. Additionally, the use of different antibodies may result in the identification of diverse cohort of patients when applied to the same study population. In a retrospective exploratory analysis of Impassion130 trial data comparing three commercially available PD-L1 assays (Ventana SP142, Ventana SP263 and Agilent 22C3 pharmDx) scored with two different scoring algorithms (ICarea and CPS), PD-L1 assays and scoring algorithms were shown to be overlapping but not entirely interchangeable in selecting patients with metastatic TNBC who are most likely to benefit from atezolizumab plus nab-paclitaxel [164]. Further studies are warranted to identify better predictive markers of response in the context of ICB-therapies [165]. Table 13.2 provides a summary of the available evidence on PD-L1 status in phase 3 clinical trials testing ICB therapy in triple-negative breast cancer (TNBC). Table 13.3 shows ongoing phase 3 trials, registered at clinicaltrials.gov.

Different types of therapies, different regimens of chemotherapy, different assays, and diverse types of tissue tested may have been responsible for the heterogeneity and inconsistency of these results. Moreover, in some trials the cut point selection has been directly dictated by the sponsor itself or even changed during the conduct of the trial following a "bottom-up" strategy which recently has received severe criticism [189, 190]. There is an urgent need to better understand the TME and help health care professionals in the search of reliable biomarkers of response to cancer treatment, such as ICB therapies, to improve patient selection as proposed by a joint action of major groups of pathologists coordinated by the TILs working group [191]. However, the evidence accumulated in these trials suggests also that there is a strong relation between PD-L1 expression and TILs in general. When looking at the patterns of PD-L1 expression,

Table 13.2 Overview of relevant phase 3 trials testing ICB therapy in breast cancer with available evidence on PD-L1 status

Acronym/ID *NCT number*	Phase *Enrollment*	BC type	Stage	Study design	Results concerning PD-L1
Triple-negative breast cancer					
IMpassion-130 *NCT02425891* [166]	Phase 3 *N = 902*	TNBC	Locally advanced Metastatic	Arm 1: First-line **Atezolizumab** + Nab-paclitaxel Arm 2: First-line *Placebo* + Nab-paclitaxel	*PD-L1 SP142 IC score cut-off 1%* Improved PFS in PD-L1+ group: hazard ratio 0.62 (95% CI 0.49–0.78, $p < 0.001$) [158] Trend to improved OS in PD-L1+ group: hazard ratio 0.71 (95% CI 0.54–0.94; exploratory, not tested) [167] *Post-hoc analysis PD-L1 SP142 IC 1%, SP263 IC 1% and 22C3 CPS > 1* [168]*:* Similar predictive value for PFS observed with all assays based on hazard rates for atezolizumab vs placebo with highest median survival benefits observed in SP142 IC >1% patients. **Conclusion on PD-L1:** **• PD-L1 SP142 validated for Atezolizumab + Nab-Paclitaxel in metastatic TNBC.** **• SP142, SP263 and 22C3 PD-L1 assays not analytically equivalent.**
IMpassion-131 *NCT03125902* [169]	Phase 3 *N = 651*	TNBC	Locally advanced Metastatic	Arm 1: First-line **Atezolizumab** + Paclitaxel Arm 2: First-line *Placebo* + Paclitaxel	*PD-L1 SP142 IC score cut-off 1%* [170] No improved PFS: hazard ratio 0.82 (95% CI 0.60–1.12, $p = 0.20$) Negative impact on OS: hazard ratio 1.55 (95% CI 0.86–2.80, trial not powered for OS) **Conclusion on PD-L1: no role of PD-L1 SP142 for Atezolizumab + Paclitaxel in metastatic TNBC**
IMpassion-031 *NCT03197935* [171]	Phase 3 *N = 324*	TNBC	Early BC	Arm 1: Neoadjuvant **Atezolizumab** + chemotherapy Arm 2: Neoadjuvant *Placebo* + chemotherapy	*PD-L1 SP142 IC score cut-off 1%* [163] Improved pCR rate for Atezolizumab arm (58% vs 41%, $p = 0.0044$) No difference in pCR rate for PD-L1 positive vs negative • PD-L1+: 69% vs 49%, diff. 20% (95% CI 6–27%, $p = 0.021$, not significant). • PD-L1-: 48% vs 34%, diff. 13% (95% CI 4–35%). Trial not powered for EFS, DFS, or OS and results are immature **Conclusion on PD-L1: Role of PD-L1 SP142 for Atezolizumab in early TNBC not demonstrated (similar to KEYNOTE-522)**
KEYNOTE-119 *NCT02555657* [172]	Phase 3 *N = 622*	TNBC	Locally advanced Metastatic	Arm 1: Pembrolizumab Arm 2: chemotherapy	*PD-L1 22C3 CPS cut-off 10* [173] No significant improvement of OS: hazard ratio 0·78 (95% CI 0·57–1·06, $p = 0·057$) PFS is not a primary endpoint *PD-L1 22C3 CPS cut-off 1* [173] No significant improvement of OS: hazard ratio: 0·86 (95% CI 0·69–1·06, $p = 0·073$) PFS is not a primary endpoint **Conclusion on PD-L1: Role of PD-L1 22C3 not demonstrated for Pembrolizumab monotherapy in metastatic TNBC**

Table 13.2 (continued)

Acronym/ID *NCT number*	Phase *Enrollment*	BC type	Stage	Study design	Results concerning PD-L1
KEYNOTE-355 *NCT02819518* [174]	Phase 3 *N = 882*	TNBC	Locally advanced Metastatic	Arm 1: First-line **Pembrolizumab** + chemotherapy Arm 2: First-line *Placebo* + chemotherapy	*PD-L1 22C3 CPS cut-off 10* [159] Improved PFS in PD-L1+ group: hazard ratio 0.65 (95% CI 0.49–0.86, $p = 0.0012$) OS assessment ongoing *PD-L1 22C3 CPS cut-off 1* [159] Improved PFS in PD-L1+ group: hazard ratio 0.74 (95% CI 0.61–0.90, $p = 0.0014$, not significant) OS assessment ongoing **Conclusion on PD-L1:** **• Role of PD-L1 22C3 demonstrated for Pembrolizumab in metastatic TNBC** **• Significant improvement of PFS from cut-off CPS 10.**
KEYNOTE-522 *NCT03036488* [175]	Phase 3 *N = 1174*	TNBC	Neoadjuvant	Arm 1: Neoadjuvant **Pembrolizumab** + Chemotherapy and adjuvant **Pembrolizumab** Arm 2: Neoadjuvant *Placebo* + Chemotherapy and adjuvant *Placebo*	*PD-L1 22C3 CPS cut-off 1/10* [162] Improved pCR rate for Pembrolizumab arm (64.8% vs 51.2%, $p < 0.001$) No difference in pCR rate for PD-L1 positive vs negative • PD-L1+: 68.9% vs 54.9%, diff. 14.2% (95% CI 5.3–23.1%) • PD-L1−: 45.3% vs 30.3%, diff. 18.3% (95% CI 3.3–36.8%) **Conclusion on PD-L1: Role of PD-L1 22C3 for Pembrolizumab in early TNBC not demonstrated** **(similar to IMpassion-030)**

it has been observed that PD-L1 expression in BC is predominant in immune cells rather than in tumor cells [165, 192]. In addition, evidence coming from mouse experiments suggests that a sufficient T cell infiltration is essential for response to PD-L1 blockade [193]. In general, high levels of TILs are strongly related to high PD-L1 expression, suggesting that TILs may be a useful surrogate for activated cytotoxic T cells. Early biomarkers of response to ICB have been investigated in the the phase 0, window of opportunity trial, Biokey. In this study, a single dose of 200 mg Pembrolizumab was administered to 54 patients in the window between time of diagnosis and surgery, either in the adjuvant or in the neoadjuvant setting. All molecular subtypes were allowed. Fresh tumor tissue and blood were collected from these patients before and 6–14 days after pembrolizumab administration. In the post-treatment samples after one single administration of pembrolizumab, significant clonotype changes in the T-cells and rearrangement of the T-cell receptor were observed, suggesting activity of the drug even after one single dose [194]. Importantly, these changes were also associated with a large shift in TILs abundancy. Notably, these results were in line with the results described in the KEYNOTE-173, in which after one dose of single-agent pembrolizumab, TILs count increased significantly as compared to baseline values, predicting pCR. In this study, high levels of PD-L1 expression and TILs were generally related to higher rates of pCR. However, because of the lack of a control arm without pembrolizumab the predictive role of TILs remains uncertain in this study [11]. In the KEYNOTE-086 patients defined as responders based on the overall response rate under pembrolizumab showed greater levels of sTILs as compared to non-responders [143, 195]. The KEYNOTE-119 trial suggested a potential role of sTILs in predicting response to pembrolizumab in a cohort of heavily pre-treated advanced TNBC patients. In this study, sTILs, both continuous and categorical with a cutoff at 5%, were significantly associated with all clinical outcomes only in the immunotherapy arm [173]. In the biomarker analysis of the IMPASSION-130 study, tumors considered as TILs+ (i.e., with a predefined cut point ≥10% sTILs; $n = 284/892$) were also PD-L1+ (i.e., IC score ≥ 1%) in almost 67% of the cases ($n = 190/284$) showing a statistically significant correlation. In the comparator arm without Atezolizumab, sTILs+ tumors did not influence the survival outcomes. Only patients exhibiting TILs+ and PD-L1 expression showed the longest improvement in progression free survival, leading to the conclusion that sTILs do not provide additional predictive value beyond that observed with PD-L1+ in patients under ICB therapy [196]. Conversely based on the analysis of the hazard ratios of benefit to atezolizumab one can speculate that in the absence of access to a PD-L1 assay, stromal TILs values of >10% assessed on H&E are able to identify a (smaller) subgroup of patients deriving benefit from ICB therapy with Atezolizumab. In a phase I/II trial testing the efficacy of the

Table 13.3 Overview of ongoing phase 3 trials testing ICB therapy in breast cancer with available evidence on PD-L1 status

Acronym/ID *NCT number*	Phase *Enrollment*	BC type	Stage	Study design	Results concerning PD-L1
Triple-negative breast cancer					
EL1SSAR *NCT04148911* [176]	Phase 3 *N = 180*	TNBC	Locally advanced Metastatic	Arm 1: **Atezolizumab** + Nab-Paclitaxel Arm 2: *Placebo* + Nab-Paclitaxel	*No results available yet (October 2024)*
GeparDouze *NCT03281954* [177]	Phase 3 *N = 1520*	TNBC	Early BC	Arm 1: Neoadjuvant chemotherapy + **Atezolizumab** followed by adjuvant **Atezolizumab** Arm 2: Neoadjuvant chemotherapy + *Placebo* followed by adjuvant *Placebo*	*No results available yet (December 2023)*
KEYLYNK-009 *NCT04191135* [178]	Phase 3 *N = 932*	TNBC	Early BC	Arm 1: Induction **Pembrolizumab** + chemotherapy followed by Olaparib + **Pembrolizumab** Arm 2: Induction **Pembrolizumab** + chemotherapy followed by chemotherapy + **Pembrolizumab**	*No results available yet (January 2026)*
IMpassion-132 *NCT03371017* [179]	Phase 3 *N = 572*	TNBC	Locally advanced Metastatic	Arm 1: **Atezolizumab** + chemotherapy Arm 2: *Placebo* + chemotherapy	*No results available yet (January 2023)*
IMpassion-030 *NCT03498716* [180]	Phase 3 *N = 2300*	TNBC	Early BC	Arm 1: Adjuvant **Atezolizumab** + Anthracycline/Taxane-Based Chemotherapy Arm 2: Adjuvant *Placebo* + Anthracycline/Taxane-Based Chemotherapy	*No results available yet (January 2022)*
NeoTRIPaPDL1 *NCT02620280* [181]	Phase 3 *278*	TNBC	Early BC	Arm 1: **Atezolizumab** + Carboplatin + Nab-Paclitaxel Arm 2: *Placebo* + Carboplatin + Nab-Paclitaxel	*Failed/No results available yet?*
SHR-1210-III-318 *NCT04335006* [182]	Phase 3 *N = 780*	TNBC	Locally advanced Metastatic	Arm 1: **Camrelizumab** + Nab-paclitaxel + Apatinib Arm 2: **Camrelizumab** + Nab-paclitaxel Arm 3: *Placebo* + Nab-paclitaxel	*No results available yet (January 2025)*
SHR1210-III-322 *NCT04613674* [183]	Phase 3 *N = 581*	TNBC	Early BC	Arm 1: Neoadjuvant **Camrelizumab** + chemotherapy Arm 2: Neoadjuvant *Placebo* + chemotherapy	*No results available yet (July 2023)*
TORCHLIGHT *NCT04085276* [184]	Phase 3 *N = 660*	TNBC	Locally advanced Metastatic	Arm 1: All lines Toripalimab + Nab-Paclitaxel Arm 2: All lines Placebo + Nab-Paclitaxel	*No results available yet (February 2022)*
HER2-amplified breast cancer					
IMpassion-050 *NCT03726879* [185]	Phase 3 *N = 454*	HER2 + BC	Early BC	Arm 1: Neoadjuvant Doxorubicin + cyclophosphamide + **Atezolizumab** followed by paclitaxel + Trastuzumab + Pertuzumab Arm 2: Neoadjuvant Doxorubicin + cyclophosphamide + *Placebo* followed by paclitaxel + Trastuzumab + Pertuzumab	*No results available yet (February 2021?)*
KATE3 *NCT04740918* [186]	Phase 3 *N = 350*	HER2+ BC	Locally advanced Metastatic	Arm 1: Trastuzumab Emtansine + **Atezolizumab** Arm 2: Trastuzumab Emtansine + *Placebo*	*No results available yet (May 2024)*
ER-positive HER2-negative breast cancer					
AMBITION *NCT04732598* [187]	Phase 3 *N = 280*	ER+/ HER2- BC	Locally advanced Metastatic	Arm 1: Bevacizumab + Paclitaxel + **Atezolizumab** Arm 2: Bevacizumab + Paclitaxel + *Placebo*	*No results available yet (June 2025)*
KEYNOTE-756 *NCT03725059* [188]	Phase 3 *N = 1140*	ER+/ HER2- BC	Early BC	Arm 1: Pembrolizumab + neoadjuvant chemotherapy + adjuvant endocrine therapy Arm 2: Placebo + neoadjuvant chemotherapy + adjuvant endocrine therapy	*No results available yet (January 2031)*

anti PD-1 antibody durvalumab in the neoadjuvant setting of TNBC patients, higher levels of PD-L1 expression by IHC (Ventana SP263 assay) were numerically associated with higher pCR rates as compared to PD-L1 negative tumors under ICB therapy. However, this difference was statistically not significant. On the opposite high stromal TILs by H&E were statistically significantly associated with higher pCR rates under ICB therapy. Higher TILs were also associated with higher PD-L1 expression levels [197]. Table 13.4 provides a summary of the current evidence of the potential clinical utility of TILs in BC patients treated with ICB therapies.

Table 13.4 Overview of clinical trials concerning ICB therapy in which sTILs are investigated

Acronym/ID *NCT number*	Phase *Enrollment*	BC type	Stage	Study design	Nature testing	sTIL cut-off	Results concerning sTIL
IMpassion-130 *NCT02425891*	Phase 3 *N = 900*	TNBC	Locally advanced Metastatic	*Arm 1*: First-line **Atezolizumab** + Nab-paclitaxel *Arm 2*: First-line *Placebo* + Nab-paclitaxel	*Retrospective*	10%	*sTIL in post-hoc biomarker analysis* [198] sTIL positive samples are associated with PD-L1 expression (Fisher's exact test, $p < 0.001$) Combo of sTIL positive and PD-L1 expression showed best improvement of PFS sTIL ≥10% alone (regardless of PD-L1 status) still predictive for PFS benefit of atezolizumab **Conclusion on sTIL: sTIL ≥ 10% predictive for benefit of atezolizumab as single marker or combined with PD-L1 testing.**
KEYNOTE-119 *NCT02555657*	Phase 3 *N = 622*	TNBC	Locally advanced Metastatic	*Arm 1*: Pembrolizumab *Arm 2*: chemotherapy	*Retrospective*	5% (median)	*TIL continuous percentage* [199] TIL levels are higher in responders than in non-responders (significant) TIL associated with all clinical outcomes ($p < 0.05$) Improved overall survival: hazard ratio 0.75 (95% CI0.59–0.96, $p = 0.0001$) TIL vs CPS: moderate correlation (0.45); multivariate analysis showed independent predictive value **Conclusion on sTIL: high sTIL levels are associated with better clinical outcome in case of Pembrolizumab therapy**
KEYNOTE-173 *NCT02819518*	Phase 1b *N = 882*	TNBC	Early BC	6 cohorts of neoadjuvant regimes with • Pembrolizumab. • (Nab-)Paclitaxel. • Carboplatin. • Doxorubicin. • Cyclophosphamide.	Prospective	/	*sTIL continuous percentage* [11] Trend toward Increase in sTIL after first administration of pembrolizumab High sTIL pre-treatment predicts pCR: 40% (95%CI 10–75%) vs 10% (95%CI 5–38%), $p = 0.0091$ Increase in sTIL on-treatment predicts pCR: 65% (95%CI 5–86%) vs 25% (95%CI 3–60%), $p = 0.0097$ sTIL and PD-L1 22C3 expression are significantly correlated: pre-treatment ρ 0.622 ($p < 0.0001$), on-treatment ρ 0.626 ($p < 0.0001$) **Conclusion on sTIL:** **• sTIL and on-treatment sTIL increase help identify responders,** **• sTILs are correlated with PD-L1 expression,** **• Unclear if sTIL and PD-L1 expression are independent predictive or prognostic biomarkers.**
KEYNOTE-086 *NCT03036488*	Phase 2 *N = 170*	TNBC	Locally advanced Metastatic	*Cohort A*: **Pembrolizumab** monotherapy, any PD-L1 expression, previously treated *Cohort B*: **Pembrolizumab** monotherapy, PD-L1 positive, previously treated	Retrospective	Median Cohort A: 17.5% Cohort B: 5%	*sTIL dichotomized by median* [200] Responders have higher levels of sTIL compared to non-responders: 10% (95%CI 7.5–25%) vs 5% (95%CI 1–10%) Improved ORR for TIL more than median: odds ratio 1.26 (95% CI 1.03–1.55, $p = 0.01$) Improved DCR: odds ratio 1.22 (95% CI 1.02–1.46, $p = 0.01$) PD-L1 22C3 is significantly correlated with sTIL% ($\rho = 0.4962$, $p < 0.001$) **Conclusion on sTIL: sTIL can help identify metastatic TNBC amenable for pembrolizumab monotherapy**

(continued)

Table 13.4 (continued)

Acronym/ID *NCT number*	Phase *Enrollment*	BC type	Stage	Study design	Nature testing	sTIL cut-off	Results concerning sTIL
Panacea *NCT02129556*	Phase 1b-2	HER2	Locally advanced Metastatic	Trastuzumab + **Pembrolizumab**	Retrospective	Continuous	*sTIL continuous percentage* [142] Correlation with ORR ($p = 0.006$) Correlation with DCR ($p = 0.0006$) **Conclusion on sTIL: sTIL levels are associated with ORR and DCR**
KATE-2 *NCT02924883*	Phase 2	HER2	Locally advanced Metastatic	*Arm 1*: **Atezolizumab** + TDM1 *Arm 2*: *Placebo* + TDM1	Retrospective	5%	*sTIL dichotomized by cut-off* [201] High sTIL associated with improved PFS: hazard ratio 1.43 (95% CI 0.51–4.01) vs 0.55 (95% CI 0.26–1.12) **Conclusion on sTIL: high sTIL levels are associated with better clinical outcome in case of Atezolizumab + T-DM1 therapy**
GeparNuevo *NCT02685059*	Phase 2	TNBC	Neoadjuvant	*Arm 1*: **Durvalumab** followed by **Durvalumab** + Nab-Paclitaxel, followed by **Durvalumab** + Epirubicin + Cyclophosphamide *Arm 2*: *Placebo* followed by *Placebo* + Nab-Paclitaxel, followed by *Placebo* + Epirubicin + Cyclophosphamide	Retrospective	10% 60%	*sTIL dichotomized by cut-offs* [202] High sTIL associated with improved pCR rate: OR 1.23 (95% CI 1.04–1.6, $p = 0.019$) vs OR = 1.39 (95% CI 1.12–1.74, $p = 0.003$) Pre-treatment sTIL not predictive for Durvalumab benefit Increase in sTIL on-treatment associated with Durvalumab benefit **Conclusion on sTIL:** **• high sTIL levels are associated with improved pCR rate regardless of Durvalumab benefit,** **• Increase in on-treatment sTIL associated with Durvalumab benefit.**
NeoTRIPaPDL1 *NCT02620280*	Phase 3	TNBC	Neoadjuvant	*Arm 1*: **Atezolizumab** + Carboplatinum + Nab-paclitaxel *Arm 2*: *Placebo* + Carboplatinum + Nab-paclitaxel	Retrospective	40%	*sTIL dichotomized by cut-off* [203] High sTIL associated with improved pCR rate: • Atezolizumab arm: pCR rate 71.43% vs. 28.07% ($p = 0.001$). • Placebo arm: pCR rate 63.16% vs. 33.90% ($p = 0.009$). **Conclusion on sTIL: high sTIL levels are associated with higher pCR rates**
GIADA *NCT04659551*	Phase 2	Luminal B	Neoadjuvant	Epirubicin + Cyclophosphamide followed by endocrine therapy + **Nivolumab**	Retrospective	Continuous	*sTIL continuous percentage* [204] sTIL levels associated with pCR rates ($p = 0.001$) **Conclusion on sTIL: sTIL levels are associated with pCR rates**

More recently ICB therapy has been tested also in HER2+ and luminal BC. In the PANACEA study which included advanced HER2+ BC patients a correlation between PD-L1+ and higher level of TILs has been observed in patients showing objective response as well as in those with stable disease. The lack of a control arm prevents from drawing final conclusions about the role of TILs as predictive marker of response [142]. In the KATE2 trial, the combination of either TDM1 + placebo or TDM1 + atezolizumab showed only borderline significant association between higher TILs and benefit from the combination therapy [201]. In the GIADA trial 43 luminal-B-like early BC patients were randomized to receive, in the neoadjuvant setting, anthracycline-based induction chemotherapy followed by the combination of nivolumab plus endocrine therapy. Higher level of sTILs was associated with pCR, while cytotoxic chemotherapy induced increase in TILs value, increase in cytotoxic T cells, and decrease in regulatory T cell in the post-treatment samples as compared to baseline [204].

Part 3: Implementation of International TIL Scoring Guidelines

Summary of the Scoring Guidelines

As mentioned above, the most widely spread methodology to assess TILs has been developed and harmonized by the founders of the TILs-WG. This international group of academic pathologists in collaboration with expert clinical oncologists, scientists, computational pathology experts, and statisticians has set up an appropriate framework for adoption of well-validated immuno-oncology biomarkers in daily and clinical trial practice, focusing mostly on TILs. It currently has over 600 members, most of them anatomic pathologists, from 43 different countries, across 6 continents. The TILs-WGs website—www.tilsinbreastcancer.org—has had nearly 30,000 visitors so far. The wide global reach of the TILs-WG enables it to understand all different viewpoints on immuno-oncology biomarkers across the world. All authors that contributed to this chapter are member of this working group.

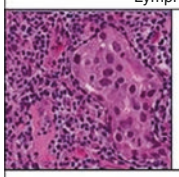
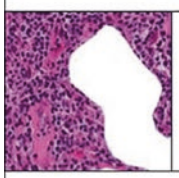
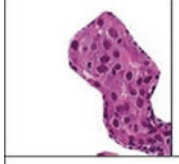
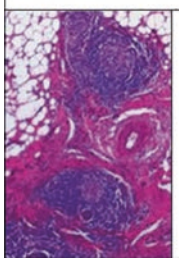
On behalf of the TILs-WG, Salgado et al. published in 2014 in Annals of Oncology this methodology and how it has been developed [14]. Table 13.5 lists these guidelines.

The TILs in these guidelines refer to stromal tumor infiltrating lymphocytes (sTILs). sTILs are located in the tumor stroma between carcinoma cells and are not in direct contact with these carcinoma cells. This is in contrast to intratumoral TILs (iTILs), which are located within the tumor nests and interact directly with the carcinoma cells. Irrespective of the biological significance of both types of TILs, sTILs seem to be more relevant for histological diagnostic purposes at the moment. sTILs are more numerously present in tumors, less variably dispersed and more easily to appreciate on H&E-stained slides, without the use of additional techniques, such as immunohistochemistry or immunofluorescence. These are important advantages for its use as an easy-to-assess and reproducible histopathological tumor parameter. Figure 13.2, adapted from Salgado et al. [14], illustrates the difference between sTILs, iTILs, and other types of lymphoid infiltrates

Table 13.5 Guidelines for assessment of tumor infiltrating lymphocytes (TILs) in breast, according to the International Immuno-Oncology Biomarker Working Group on Breast Cancer (14, By permission of Oxford University Press on behalf of the European Society for Medical Oncology)

1	TILs should be reported for the stromal compartment (= % sTILs). The denominator used to determine the % sTILs is the area of stromal tissue (i.e., area occupied by mononuclear inflammatory cells over total intratumoral stromal area), not the number of stromal cells (i.e., fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei).
2	TILs should be evaluated within the borders of the invasive tumor.
3	Exclude TILs outside of the tumor border and around DCIS and normal lobules.
4	Exclude TILs in tumor zones with crush artifacts, necrosis, regressive hyalinization as well as in the previous core biopsy site.
5	All mononuclear cells (including lymphocytes and plasma cells) should be scored, but polymorphonuclear leukocytes are excluded.
6	One section (4–5 μm, magnification ×200–400) per patient is currently considered to be sufficient.
7	Full sections are preferred over biopsies whenever possible. Cores can be used in the pretherapeutic neoadjuvant setting; currently no validated methodology has been developed to score TILs after neoadjuvant treatment.
8	A full assessment of average TILs in the tumor area by the pathologist should be used. Do not focus on hotspots.
9	The working group's consensus is that TILs may provide more biological relevant information when scored as a continuous variable, since this will allow more accurate statistical analyses, which can later be categorized around different thresholds. However, in daily practice, most pathologists will rarely report for example 13.5% and will round up to the nearest 5%–10%, in this example thus 15%. Pathologist should report their scores in as much detail as the pathologist feels comfortable with.
10	TILs should be assessed as a continuous parameter. The percentage of sTILs is a semiquantitative parameter for this assessment, for example, 80% sTILs means that 80% of the stromal area shows a dense mononuclear infiltrate. For assessment of percentage values, the dissociated growth pattern of lymphocytes needs to be considered. Lymphocytes typically do not form solid cellular aggregates; therefore, the designation "100% sTILs" would still allow some empty tissue space between the individual lymphocytes.
11	No formal recommendation for a clinically relevant TIL threshold(s) can be given at this stage. The consensus was that a valid methodology is currently more important than issues of thresholds for clinical use, which will be determined once a solid methodology is in place. Lymphocyte predominant breast cancer can be used as a descriptive term for tumors that contain "more lymphocytes than tumor cells." However, the thresholds vary between 50% and 60% stromal lymphocytes.

a

Morphology	Definition and biological relevance	Diagnostic relevance
Lymphocyte-predominant breast cancer (LPBC)		
	Working category to describe tumors with "more lymphocytes than tymor cells".	Definitions vary across studies with stromal TILs of 50–60% used as a threshold. LPBC can be used for predefined subgroup analyses and for description of tumors with a particularly high immune infiltrate, however, keep in mind that TILs are a continuous parameter and the threshold for LPBC is still arbitrary.
Stromal TILs		
	Indicator of increased accumulation of immune-cells in tumor tissue	Stromal TILs have been shown to be predictive for increased response to neoadjivant chemotherapy as well as improved outcome after adjuvant chemptherapy. Based on current data, this parameter is the best parameter for characterization of TILs.
Intratumoral TILs		
	TILs with direct cell-cell contact with carcinoma cells, might be an indicator of direct cell-based anti-tumor effects.	Several studies have shown that intratumoral TILs and more difficult to evaluate and do not provide additional predictive/prognostic information compared to stromal TILs.
TILs at the invasive margin		
The localization of TILs are the invasive edge is included in the evaluation approach presented in this guideline.		For breast cancer there are no studeis with a separate evaluation of TILs at the invasive edge. For practical purposes, the reliable evaluation of the invasive edge might be difficult when using core biopsies in the neoadjuvant setting.
Tertiary lymphoid structures (TLS)		
	Typically localized in the surrounding area of the tumor, TLS might be localized in normal tissue directly adjacent to the tumor, consisting of a T cell zone next to a B cell follicle, often with germinal centers.	While these structures may be important for the biology of tumor-immune reactions, they are not yet optimized for non-research based assessments. The main problem is that TILs have a spatial heterogeneity and are principally localized in areas surrounding the tumor. They might not be in the plane of the tissue section that is being evaluated, in particular when using core biopsies. Furthermore, it might be difficult to distinguish lymphoid aggregates from true TILs, in particular when the germinal center is not in the plane of the section.

b

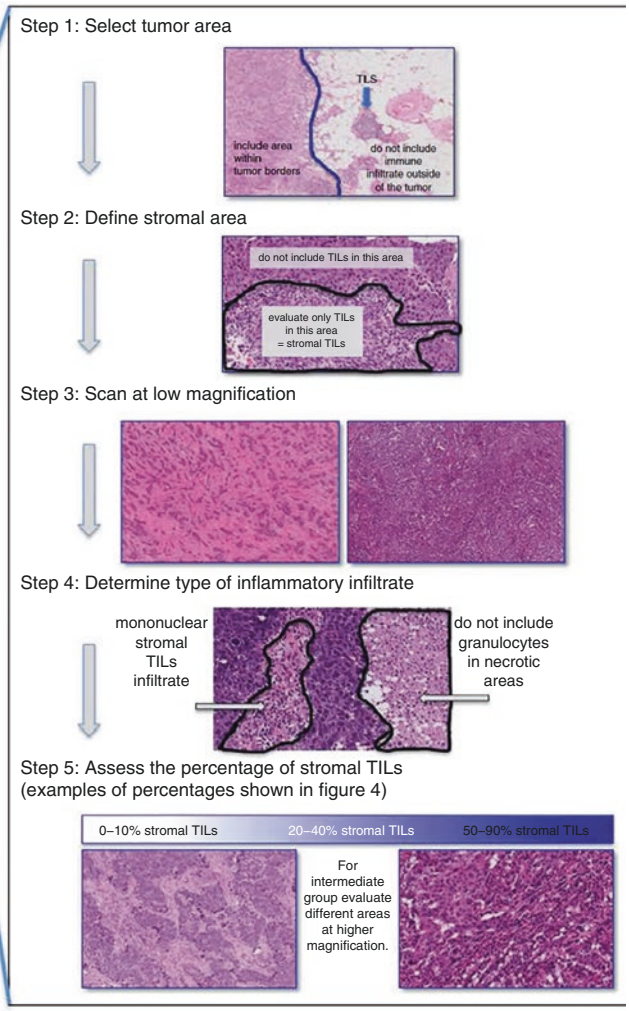


Fig. 13.2 (**a**) Morphology, definitions, biological and diagnostic relevance of the different immune infiltrates found in breast cancer. (**b**) Standardized approach for sTILs evaluation in breast cancer (14, By permission of Oxford University Press on behalf of the European Society for Medical Oncology)

and gives an overview on the scoring method for sTILs. For a more detailed discussion, we refer to the original publication.

Guidelines Include TIL Scoring

International expert committees like those of the St. Gallen Breast Cancer Conference and European Society of Medical Oncology recognize in their latest published recommendations the prognostic importance of TILs in TNBC, encouraging reporting in daily practice, while cautioning that TILs should not be used solely to determine treatment options, as treatments are governed by stage. It needs to be emphasized that the level IB evidence of the TILs as a prognostic factor in TNBC does not mean that TILs should be used as a binary variable for treatment selection, either for de-escalation of chemotherapy or for immunotherapeutic approaches. Binary use of any biomarker, to decide to treat or not to treat the patient based on the result of the biomarker, depends on prospective randomized controlled trials designed to address the utility of the biomarker at predefined cut-offs, thus on level IA-evidence. This level IA-evidence is not yet present for the TILs, suggesting that TILs should be used in conjunction with other prognostic variables such as tumor size and lymph node status to inform the clinician on the outcome of the patient. The clinician then informs the patient, to take in mutual agreement with the patient, the most optimal treatment. Clinical trials where TILs are used as an inclusive biomarker to decide on treatment are in development. In daily practice, some clinicians are still reluctant to include the TILs in their daily practice because there is no level IA evidence, however most other prognostic factors used in breast cancer practice do not have that level of evidence either. Treatments were escalated based on the worse outcome with

the prognostic factors and were not designed to assess prospective in a binary value the value of that biomarker. For predictive purposes, two phase 3 clinical trials, namely KEYNOTE-119 [173] and IMpassion130 [196], showed that TILs predict benefit to immunotherapy, so conceptually level of evidence IB, yet because the TILs are used as a predictive biomarker, a binary approach is needed, hence level of evidence IA. So, TILs should not be used for immunotherapeutic treatment decisions, and in this context, TILs can be used to support PD-L1-assessment, as if there are no TILs, any PD-L1 assay is likely to be negative, while if many TILs are present, any PD-L1-assay is likely to be positive.

This interpretation is important, as some clinicians in daily practice do not consider the TILs as a binary variable, since it has no level IA-evidence so they do not ask for it, while others recognize that TILs can be used together with other prognostic variables, and hence they ask for it. This difference is also informative for discussion at international expert committees, as it all depends on how the question about the use of TILs counts is posed. We believe that experts should answer to the question whether there is enough evidence to use TILs quantities in combination with other features, instead of answering to questions about the use of TILs for treatment decisions. Formal endorsement of the use of TILs by pathology societies is also variable and fragmented depending on the country.

Practical Aspects of the Implementation of TILs in Breast Cancer

Section "Summary of the Scoring Guidelines" illustrates in detail the scoring guidelines for sTILs as proposed by the TILs-WG. In this section, we provide a practical guide for sTILs scoring and interpretation in clinical practice, challenges and pitfalls pathologists may encounter and we give an overview of training resources that are freely available.

Pitfalls When Scoring sTILs and Their Remediation

In 2015, the TIL-WG published the first practical guideline to evaluate sTILs in H&E stained tissue section of breast cancer specimens [14]. Subsequently, several reproducibility studies evaluating the robustness of this method among pathologists were conducted, both by the TIL-WG and other research groups [89, 113, 114, 205–207]. While these studies showed on average acceptable to—in some studies—excellent interobserver reproducibility of this method, these pivotal reproducibility studies also identified sources of variability and difficulties pathologists may face when applying sTILs evaluation in their daily practices. A systematic analysis of such sources of variability and recommendations on how to handle these was published by Kos et al. in 2020 [16]. For this study, data were analyzed from three different RING trials conducted by the TIL-WG. For each of these RING studies sTILs were evaluated by 6–32 dedicated pathologists on H&E stained slides (core needle biopsies or whole tumor sections) of 60–100 invasive breast carcinomas [89, 114]. Based on the highest variation between individual pathologist's sTILs scores, 4 categories of pitfalls in sTILs assessment were identified. Here, we briefly discuss each of these categories and provide some tips and tricks on how to cope with them when evaluating sTILs. Extensive description can be found in the original paper by Kos et al. [16].

1. *Heterogeneity in sTILs distribution:* Heterogeneity in sTILs distribution was identified as the most important factor contributing to variability in pathologist's sTILs scores. When assessing sTILs, all peri- and intratumoral stroma associated with invasive carcinoma is to be included in the denominator. When sTILs are heterogeneously distributed within this stroma, the pathologist has to average the different density levels of sTILs relative to the area they occupy into a single score.

   Frequent patterns of heterogenous distribution of the immune infiltrate in a tumor that can render sTILs evaluation challenging, consist of increased density of sTILs at the invasive front as opposed to the center of the tumor, when a tumor is composed of variably spaced tumoral cell nests associated with sTILs, or abundant stroma that is sparse in lymphocytic infiltrate in between. In such situations, it is advised to evaluate sTILs in multiple (at least three) fields of view that are representative of the overall pattern of sTILs observed at low magnification and average the results into one global sTILs score rather than trying to eye-ball a single global sTILs score all at once.
2. *Technical factors:* A lot of different technical factors such as fixation (mainly underfixation), microtomy, and crush artifacts, fading of H&E stainings over time and—as more and more labs are adopting digital pathology—scanning focus errors can all contribute to poor reproducibility of sTILs evaluation. Keeping such variables as much as possible under control is a primary focus of every pathology lab. When artifacts are only focally present, sTILs should be scored in unaffected areas and in general pathologists should have a low threshold not to evaluate prognostic or predictive biomarkers when reliable assessment is hampered by technical factors.
3. *Problems with identifying area or cells of interest:* As outlined above, for the tumor area all peri- and intratumoral tumor-associated stroma should be taken into account and included in the denominator when evaluating sTILs. An exception to this rule is the presence of a central hyalinized scar or fibrotic focus, which is excluded from sTILs scoring. Challenging cases can be encountered

when DCIS or pre-existent benign structures are present with the boundaries of an invasive carcinoma. Lymphocytic infiltrate clearly associated with DCIS or benign structures should not be included in the sTILs score. In cases with a very heterogenous composition of invasive carcinoma, DCIS and pre-existent normal structures, a similar representative field-of-view scoring approach as described above focusing on invasive carcinoma can be helpful.

With respect to the cells of interest, by definition sTILs evaluation is restricted to loosely organized infiltrate of lymphocytes and plasma cells in a tumor. Cases of invasive carcinoma with abundant infiltrate of histiocytes, neutrophils, or abundant presence of apoptotic cells—which can at low magnification mimic lymphocytes—can be challenging to score. These cases often require more detailed evaluation of the immune infiltrate at high magnification in different areas of the tumor before assessing the sTILs score. In addition, confined dense lymphoid aggregates and organized lymphoid structures such as tertiary lymphoid structures (TLS) are also excluded from the sTILs score.

4. *Cases with little evaluable stroma:* A final category of cases in which sTILs evaluation can be challenging are those with only limited stroma present within the tumor. This can be encountered in highly cellular tumors with a high tumor-stroma ratio, micropapillary carcinomas, mucinous tumors with only slender fibrovascular cores in between the mucus lakes or tumors with abundant necrosis obscuring fibrous stroma. In such cases, even an apparently paucicellular stromal immune infiltrate in absolute numbers can result in paradoxically high sTILs scores. There is currently no formal guideline defined on minimal sample requirements for scoring sTILs, neither in terms of minimal number of tumor cells nor in terms of minimal amount of evaluable tumor-associated stroma to be present. As a general rule of thumb, pathologists should use judgment. Also, important to keep in mind when scoring sTILs in samples with little stroma is that iTILs, i.e. immune cells that are present with the epithelial cell nests, should not be included in the sTILs score.

Similarly O'Loughlin and colleagues found that factors contributing to discrepancies between pathologist were intratumoral heterogeneity of TILs, necrosis, biopsy fragmentation, tumor cellularity, and difficulties in the identification of the tumor border [113]. Figure 13.3 illustrates some of the most frequently encountered pitfalls.

Available Resources for Pathologists

To aid pathologists and researchers in getting familiar with how sTILs were scored in clinical trials that have shown clinical validity and potential clinical utility of this biomarker, the TIL-WG has developed a wealth of educational tools and resources that are freely available via their website www.tilsinbreastcancer.org. On this website, an extensive training section can be found where literature references, video tutorials, tutorial slide decks with step-by-step explanations on how to score TILs in different cancer types (invasive breast cancer, DCIS, and breast cancer after neoadjuvant chemotherapy, but also melanoma, NSCLC adenocarcinoma, endometrial carcinoma, urothelial carcinoma, and colorectal carcinoma) and sheets of reference images that can be used as a visual guide when scoring sTILs are centralized. Specific guidance on how to handle challenging cases can be found in a separate section on the website at www.tilsinbreastcancer.org/pitfalls.

The use of a well-calibrated set of reference images together with a structured representative sampling approach for heterogeneous cases has been shown to be the most effective way to improve reproducibility and to reduce scaling differences among pathologists. This was clearly illustrated in one of the RING trials conducted by the TILs-WG in which ICC values amongst an identical group of 28 pathologists improved considerably from 0.71 (95% CI 0.63–0.79) to 0.89 (95% CI 0.85–0.92) after the implementation of a software tool that guided pathologists to select different areas for sTILs evaluation and provided direct visual feedback for provided sTILs values [114].

In addition, the website hosts an interactive scoring platform (available through the link "Teach yourself to score TILs") with two functionalities. First, a large collection of training images that have been assigned a consensus sTILs score by expert members of the TIL-WG is available. Here, pathologists can run training sessions in which these images are randomly presented, sTILs can be scored with or without the use of reference images and a progress report of training status summarizing the concordance c.q. deviation of assigned scores compared to the reference scores can be generated. A second functionality of this interactive tool provides the users with the possibility to upload snapshot images (ideally .jpeg or .png images of 20× fields-of-view) from own clinical cases and compare these images to a reference set of images of invasive breast cancer with verified sTILs. Multiple images per case can be uploaded and can then be assigned a sTILs score with 10% increments after which a summary report with automatic calculation of an average sTILs score for the entire case can be generated. This tool is particularly helpful in case of heterogenous distribution of sTILs where eyeballing a single global sTILs sore is not recommended, as explained earlier.

Currently, projects on the development of imaging analysis tools for automated measurement of sTILs in invasive breast cancer images using machine learning techniques are ongoing [208].

Finally, during the first half of 2021, the TIL-WG, in collaboration with the Biomedical Quality Assurance Research

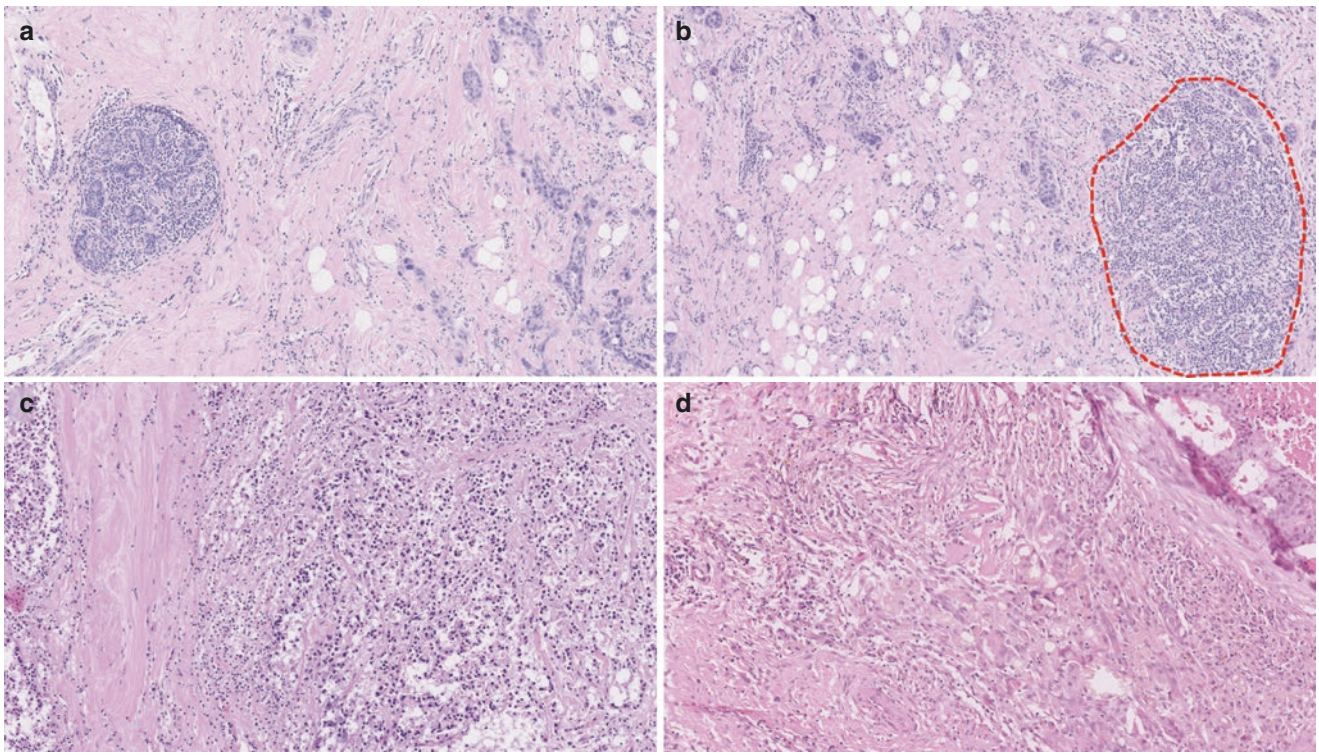


Fig. 13.3 H&E stained slides of invasive breast carcinoma specimens illustrating some frequently encountered pitfalls when scoring sTILs. All images were extracted from digitized whole slide images at 200x magnification. (**a**) sTILs are evaluated in tumor-associated stroma only. Lymphoid infiltrate clearly associated with normal lobules should be disregarded. In this field of view, consensus sTILs score within the boundaries of the invasive carcinoma was determined by two expert pathologists of the TIL-WG to be 10%. (**b**) Confined dense lymphoid aggregates and especially organized lymphoid elements such as tertiary lymphoid structures (TLS) are not included in the sTILs score. In this example, the encircled dense lymphoid aggregate should not be taken into account for the sTILs score. Consensus score for sTILs in this field of view as determined by two expert pathologists of the TIL-WG is 20%. (**c**) This case clearly illustrates the difficulties one may encounter to distinguish tumor cells from immune infiltrate when breast cancer tissue is not properly fixed. General principles of good laboratory practice for handling breast cancer specimens should be strictly adhered to (including limiting delay to fixation to less than 30 min and minimum duration of fixation for breast cancer specimens to more than 6 h as much as possible). (**d**) This image shows a confined area of inflammation with many iron macrophages consistent with a biopsy site. Apart from macrophages never to be included into the sTILs score, sTILs should always be scored outside areas of inflammation attributable to iatrogenic manipulation (biopsy sites, implantation site of radiographic markers)

Unit of the Catholic University Leuven (Leuven, Belgium) will launch an external quality assessment (EQA) scheme for sTILs in triple-negative breast cancer (TNBC) for laboratories and pathologists worldwide. In addition to the EQA scheme for sTILs, also exploratory data will be collected on the real-world implementation of PD-L1 assays and scoring algorithms in TNBC. This EQA program will be available at https://tils.agoko.be.

Choice of Sample Types, Interobserver Reproducibility, and Impact on Clinical Validity

As mentioned above, the interobserver reproducibility of the described method has been documented in multiple studies including 2 to up to 40 different pathologists evaluating biopsies or full-face sections of invasive [89, 113, 114, 205–207] carcinoma. In these studies, intraclass correlation coefficients for concordance between pathologists ranged from around 0.60 to 0.95 when evaluating sTILs as continuous variable. Measures resulting in a marked positive effect on scoring reproducibility include the use of reference images and structured representative sampling approaches for assessing cases with heterogenous sTILs distribution [114]. While these data are reassuring for the evaluation of sTILs in biomarker driven treatment decision schemes in the years to come, they also clearly highlight the need for sustained training efforts and participation in external quality assessment and reader proficiency testing programs when sTILs evaluation is introduced in clinical practice. An important question is how the observed interobserver variability affects the clinical validity and clinical utility of a biomarker, especially when discrete cut-offs are taken into consideration. In an effort to statistically defer the impact of variability in sTILs assessment on pathological complete response (pCR) prediction, the TILs-WG calculated for the neoadjuvant GeparSixto trial [209], that for any intraclass correlation coefficient for scoring concordance between pathologists (ranging from 0.6

to 0.9), comparable odds ratios for pCR prediction would be obtained [210]. Several studies specifically designed to evaluate the impact of interobserver variability in sTILs assessment on prediction of response to neoadjuvant chemotherapy in a real-life sample sets are currently underway (personal communication Dr. Van Bockstal and Dr. Callagy).

Finally, given the fact that the currently most robust data supporting clinical application of sTILs assessment are situated within the primary disease setting, a relevant question to ask is to what extent sTILs assessment in small core needle biopsies is representative for the overall immune infiltrate in the whole tumor. As previously mentioned, no minimum sample requirements for sTILs evaluation in terms of number of invasive tumor cells or amount of evaluable tumor-associated stroma that must be present in order to be able to reliable assess the sTILs have currently been defined. At least two studies have shown excellent correlation of sTILs evaluation of core needle biopsies as compared to full-face sections of the corresponding primary resection specimen, especially when multiple cores per biopsy were examined [211, 212]. In addition, in a study by Althobiti et al. no statistically significant difference could be observed in sTILs scores evaluated on slides from different tissue blocks of the same tumor [213]. Together, these data further underscore the robustness of the proposed sTILs evaluation system in heterogenous sample types.

Part 4: Novel Methods

As described above, the quantification of (s)TILs can easily be incorporated in a standard pathology examination of (breast cancer) biopsies and resection specimens. It is a good general semiquantitative histopathological measure of the immune reaction in a tumor. New developments will aim at more standard and more quantitative measurement, on the one hand, or further, more detailed, characterization of the immune response, on the other hand. The purpose of this section is to briefly describe new evolutions and technologies that are emerging in this field.

Tumor Mutational Burden

The measure of the TILs on H&E is generally regarded as a surrogate of tumor immunogenicity. However, the quantification of TILs alone may not be sufficient to capture clinically and biologically meaningful differences between the different breast cancer subtypes. From an immunological point of view, the presence of foreign antigens is crucial for the activation of the adaptive immune system and elimination of potentially harmful organisms. Several mechanisms contribute to the accumulation of mutations in cancer genes and contribute to cancer progression. This process is supposed to generate a number of neoantigens that are capable of laying the basis of the interactions with the immune system and promote immune editing [8]. The ultimate aggregate of all mutations found in a tumor is defined as tumor mutational burden (TMB). As indicated by recent studies based on whole genome sequencing analysis, some types of cancers more than others are characterized by the accumulation of higher numbers of non-synonymous mutations across the whole DNA. Melanoma and lung carcinoma are typical examples of tumor types with high TMB, microsatellite instability also attributes to high TMB to colon, while mutations in the DNA polymerase genes may contribute to hyper mutant cancer phenotypes in endometrial carcinoma [214]. Tumors with high TMB profiles seem to respond better to ICB therapies, providing proof for the concept that higher TMB is synonymous of higher antigenicity. Based on these observations pembrolizumab has been approved in a tumor agnostic manner in all cancer types showing microsatellite instability defects [215]. However, as briefly mentioned before microsatellite instability has an incidence of <2% in BC and BC are considered as "cold tumors" with poor antigenicity because of the low TMB [63, 216]. Despite this observation, ICB has also in BC the potential to become a new mainstay of treatment for at least certain patients [157, 162]. Therefore, there is an urgent need to better understand this paradox and help health care professionals in the search of reliable biomarkers of response to ICB therapy to increase patient selection [217]. In this regard recent evidence suggests that contrary to what initially thought, TMB in general is unlikely to become a pancancer marker for predicting response to ICB therapy. McGrail and colleagues found that overall response rates to ICB therapy above 20% were present only in TMB-high tumors with a positive correlation between CD8+ T cells and neoantigen load, while in the tumors lacking this type of correlation (e.g., breast, prostate, glioma, etc.) the overall response rates were <20% and were statistically significantly lower when compared to the TMB low tumors [13]. A recent meta-analysis performed by Litchfield et al. demonstrated in a pancancer study, comprising over 1000 patients, that clonal TMB has a better predictive value for the response to therapy when comparing to total TMB or subclonal TMB, suggesting that it is the early signatures in tumor development that makes a tumor sensitive for ICB [218]. Examples are the APOBEC signature, the UV-signature in melanoma, and Tobacco signature in NSCLC. The diversity of further subclonal mutational signatures would "confuse" the immune system, resulting in an ineffective response. Nevertheless, there is quite some heterogeneity between different cancer types and histologies that renders the effect small when looking at single histology type. Therefore, a multimodal approach for assessing sensitivity to ICB is more likely to be effective in choosing the

right therapy for the patient, integrating (clonal) TMB, specific TMB signatures, and immune infiltrate abundance and phenotypes [161].

CD8+ T Lymphocytes

The CD8+ T lymphocytes play a major role in the recognition of tumor-specific epitopes which are presented by antigen presenting cells. The T-cell mediated antigen recognition depends on the interaction of T cell receptor (TCR) with the antigen-major histocompatibility complex molecules. Only activated CD8+ cytotoxic T cells can efficiently kill tumor cells upon specific recognition of tumor-specific antigens [219]. A large amount of evidence accumulated in the past strongly suggest that the presence of cytotoxic CD8+ T cells is associated with longer survival rates and higher rates of pCR after neoadjuvant chemotherapy in BC patients [76, 77, 220–223]. In addition, recent evidence based on multiomics analysis suggests that estimates of CD8 + T-cells abundance may represent the most robust predictive biomarker for the response to anti PD-1/PD-L1 therapy across multiple types of cancers including breast [224]. However, the biomarker analysis of the IMpassion130 study showed that CD8+ tumors were associated with improved survival outcomes only when also PD-L1 was positive [196]. Prolonged exposure to tumor neoantigens may induce sustained expression of immune checkpoint molecules that eventually may result in a dysfunctional T-cell state or apoptotic CD8+ T cells which is not resolved even when antigens are removed (tumor immunotolerance and tumor immunosuppression) [225]. Metabolic signals coming from the tumor microenvironment or other factors that are intrinsically related to the tumor cells may contribute to the dysfunctional status of the cytotoxic T cells, explaining at least in part why ICB therapies are successful only in a minority of patients [226, 227]. In this regard, the study of specific subset of TILs may give a substantial contribution in the understanding of the involved mechanisms of immune editing. In treatment naïve TNBC important quantitative and qualitative variations in the composition of CD8+ T cells were recently observed. T cells with CD8 + CD103+ displayed a distinctive phenotype as compared to the subset of CD8 + CD103- T cells, indicating in the former group higher PD1 expression levels, ability of clonal expansion and cytotoxic activity suggesting important immune surveillance functions. Interestingly, higher levels of TILs were associated with the CD8 + CD103+ phenotype proposing a mechanistic relation between high TILs levels and good prognosis in TNBC patients [228]. In the window of opportunity trial Biokey, after one single administration of pembrolizumab, significant clonotype changes in the T-cells were observed. In particular Pembrolizumab induced expansion of the PD1+ T-cells expressing CD8 or CD4 markers. These cells showed mainly markers which suggested activation of T cells based on the expression of immune checkpoint (LAG3, HAVCR2, PDCD1), effector (IFNG, NKG7) and cytotoxic (GZMB, PRF1) markers; only a small portion of the expanded T cells showed a CD8+ effector/memory phenotype [194]. These data strongly suggest that the identification of specific subset of TILs may be important for the identification of functional markers that can improve patient selection for ICB therapies.

Spatial Heterogeneity of Immune Infiltrate in the Breast

The spatial resolution of the lymphocytes present in the tumor microenvironment can further refine the classification of BC in meaningful prognostic categories [228, 229]. In this regard, tumors can be classified as immune-inflamed, immune-excluded, and immune deserted tumors based on the quantity and the topographical localization of the immune cells in relation to stroma and tumor cells [229] (Fig. 13.4). For instance, luminal BC and special histologic subtypes of BC have been regarded as immune-excluded tumors because of the lack of intraepithelial CD8+ T cells [23, 77]. The topographical identification on H&E of lymphocytic hot spot seems also to have important implications for the prognosis of BC, as illustrated by studies performed with the aid of computational pathology [230, 231]. In an exploratory study performed on luminal B tumors across three different age groups, it has been observed that CD20+ and FOXP3+ inflammatory cells were more abundant at the periphery and in the inner part of the tumor regardless of age, respectively [232, 233]. The spatial pattern of TILs seems to be comparable when primary tumors are matched to their metastasis, suggesting in the metastasis a kind of TILs imprinting which is derived from the primary tumor [234]. Additionally, as recently suggested, the immune-excluded BC category can be further classified as margin-restricted or stroma-restricted. On the other hand, the inflamed tumors can be distinguished in stromal-intraepithelial and stromal-restricted both potentially associated with different prognostic information [228, 235]. However, the biological meaning of these categories is not fully understood yet and, importantly, studies performed on tissue microarray or with small pieces of tissue may introduce sampling bias and prevent to fully understand spatial heterogeneity in BC [236, 237].

Spatial Single Cell Technologies

A detailed map of the interactions existing between tumor cells and its surrounding microenvironment requires the availability of highly specialized technologies that can mea-

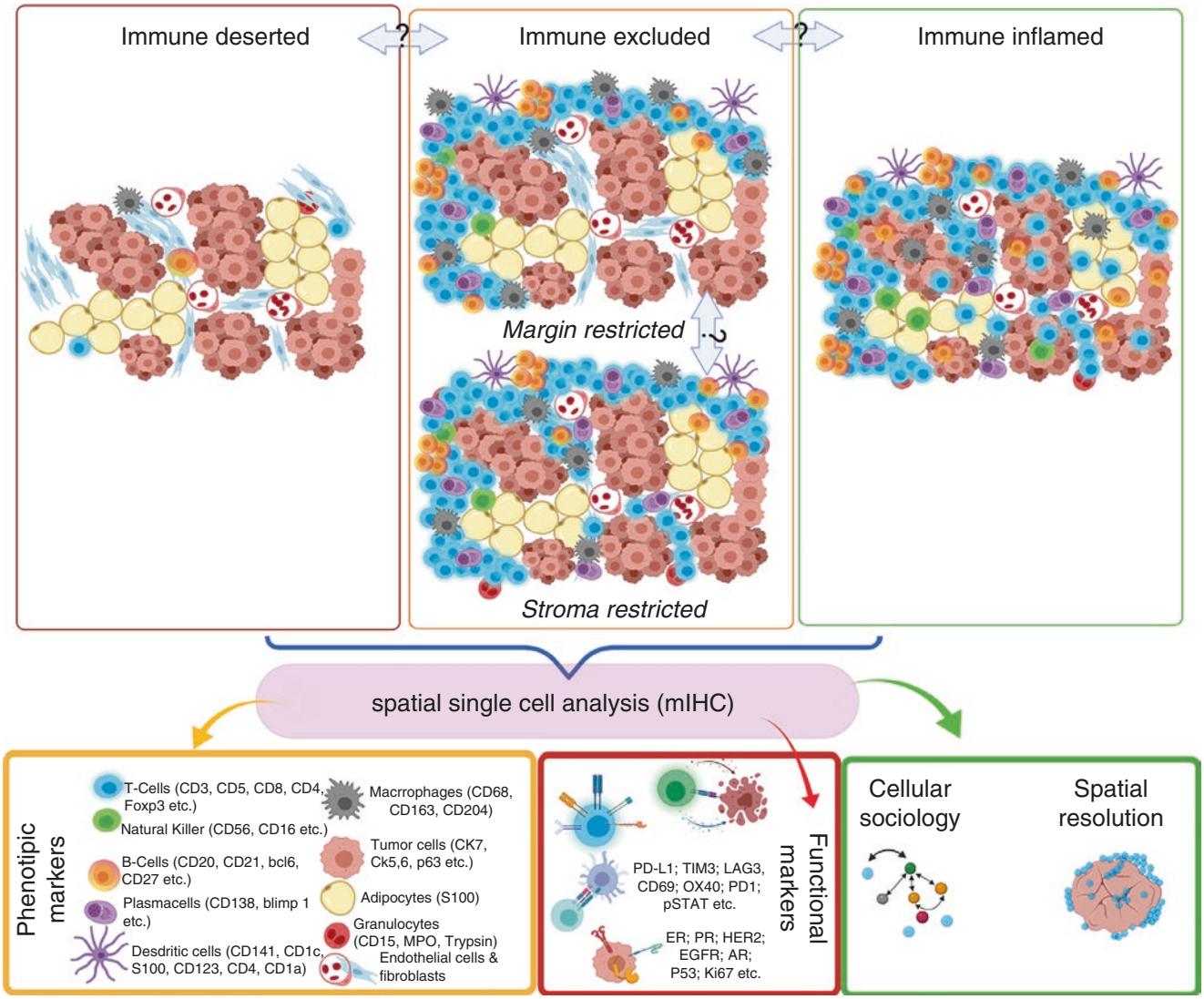


Fig. 13.4 The spatial lymphocytic phenotypes of immune infiltration in BC. BC are often divided into poorly infiltrated (immune deserted, left panel), immune excluded (central panel), and inflamed (right panel). "Immune deserted" tumors show almost total lack of lymphocytes. "Immune excluded" tumors show a lack of lymphocytes within the epithelial nests of the tumor, but lymphocytes may be present at the invasive margin (margin restricted) or spread in the whole surface but restricted only to the stroma (stroma restricted). "Inflamed tumors" show lymphocytic infiltration lymphocytic infiltration in the stroma and intratumorally. To date it is unclear whether these patterns are related to specific biological features existing between tumor cells and its microenvironment or represent different magnitude of infiltration. It is anticipated that the use of spatial single cells technologies like m-IHC will allow us to achieve four goals using one tumor section: (1) identify each single cellular component of a tumor mass (orange panel); (2) infer about the functionality of the different cells (red box); (3) understand the interactions between cells using neighborhood analysis (cellular sociology, green panel); (4) topographical mapping of all the components to resolve at the spatial level the interactions between tumor cells and its microenvironment. Figure created with BioRender.com

sure a multitude of features in single cells while maintaining their original position in a tissue. Such technologies are now becoming available, from which multiplexed immunohistochemistry (m-IHC) is the most advanced [238]. By using m-IHC, the interactions between tumor cells and its TME are preserved, overcoming the shortcomings provided by cell dissociation and cell enrichment of other single cell technologies [239].

The use of m-IHC in BC pathology is emerging as useful tool to gain better insight in the cellular composition of the tumor microenvironment, understand relationship between different cells including mechanisms of activation, and most importantly acquire spatial information. Using imaging mass cytometry, Jackson et al. recently described the complex structure of BC at single cell level. With a panel of 35 metal-labeled antibodies, used simultaneously to detect different cell populations, the researchers have been able to identify different cellular communities which were correlated to different molecular subtypes, beyond those already known. Tumors also showed some level of spatial heterogeneity in

about 60% of the cases and related to spatial heterogeneity. Interestingly, survival analysis performed on spatially identified cellular communities was able to provide strong prognostic information beyond current clinical classification [240]. Using multiplexed ion beam imaging, Keren et al. were able describe the spatial organization of inflammatory infiltrates in TNBC [235, 240]. In their experiment using simultaneously over 30 different Ab in 41 samples of TNBC patients they found two main cellular compartments: epithelial and inflammatory cell compartment. Depending on the quantity and spatial interactions between epithelial and inflammatory cells tumors were subdivided into cold (low number of inflammatory cells), mixed (heterogeneous mixture of the two compartment), and compartmentalized (physical separation of either of the two compartment). Expression of PD1 was associated with either CD8+ or CD4+ T cells in mixed or compartmentalized tumors, cold tumors were rarely observed. CD4 + PD1+ were found to be more frequently associated with the compartmentalized tumors and were spatially segregated showing additional co-expression of other immune-modulatory markers like IDO or PD-L1. Conversely, mixed tumors showed higher level of CD8 + PD1+ T cells which were more frequently admixed with tumor cells expressing IDO or PD-L1 immune-modulatory molecules. Survival analysis showed improved survival outcomes in the compartmentalized tumors as compared to the mixed ones. These findings were in contrast with those reported by Gruosso et al., however in the latter no co-expression analysis or CD4 assessment was performed [228, 235]. Alternatively, the spatial identification of CD8 + CD103+ T cells with resident memory phenotype suggests that these cells are associated with nests of epithelial cancer cells being associated with improved relapse free survival as suggested by multiplex analysis using low-plex methods with multiple cycles of staining [141, 241, 242].

Pre- and post-treatment matched samples of patients with inflammatory breast carcinoma treated with neoadjuvant chemotherapy have been studied by "first-generation" m-IHC with medium-plex panel of inflammatory ad myeloid cell lineages. Low level of macrophages in the pre-treatment biopsy was associated with higher pCR rates, while spatial analysis revealed a significant association between mast cells, CD163+ cells, and CD8+ cells in patients with residual disease, suggesting a potential mechanism of resistance [243]. In BC patients with HER2+ disease low- to medium plex m-IHC studies have been conducted as well to understand spatial immune profiling in relation to anti HER2-therapy. These studies linked the activity of trastuzumab directly to extracellular domain of HER2 and co-localization of CD8+ T cells providing further evidence for the involvement of the immune system in the mechanism of action of monoclonal Ab directed against HER2 [244]. Similarly, in another study from an independent group it was observed that HER2+ BC showing immune cells spatially interacting with tumor cells had higher responses to anti-HER2 therapies [245]. Tumors characterized by high stromal CD4+, CD8+, CD20+, and high intratumoral CD20+ immune cells showed higher rates of pCR in another analysis performed on HER2+ BC [246].

Artificial Intelligence

Another technological (r)evolution that will influence the field of pathology in general and that of TILs assessment in particular in the near future will be artificial intelligence (AI). The use of AI is currently under immense investigation (the big boom of AI is expected to happen in the next 10–15 years), and there are in fact already clinically validated algorithms for quantification or categorization of well-known histopathological biomarkers [247]. Some of these algorithms are already CE-IVD (Europe) approved. Concerning TILs, there are several methods described in the literature. The most accessible method is scoring of sTILs on H&E slides. On top of the application of TILs in H&E, more and more investigators are also trying to dissect the tumor microenvironment by using multiplex immunohistochemistry as discussed above [247]. To this date, no CE-IVD labeled algorithm for AI-based sTIL assessment exists. Yet, this topic is under thorough investigation for several types of cancer, among which breast cancer, melanoma, colorectal cancer, and lung cancer [231, 248–257]. The general principles for assessing TILs are simple. The AI-based algorithm analyzes the whole slide image via convolutional neural networks (CNN) or other another artificial neural network, differentiating tumor stroma from tumoral structures. In a next step TILs are differentiated in the stroma from other (inflammatory cells) that cannot be considered when scoring TILs. To have the most accurate result, the application needs to follow the TIL scoring guidelines as closely as possible [247].

An extensive review of computational assessment of sTIL was written by Amgad et al. in 2020 on behalf of the TILs-WG. It does not only graphically describe the aforementioned general steps, but also describes all possible approaches and methods used in literature for automated sTIL evaluation. The author divides the overview into H&E based approaches and immunohistochemistry based approaches. Table 13.6 is adapted from this data to give an overview of all possible methods and approaches and their strengths and weaknesses as reported by the authors [208].

Although AI is a promising addition to pathological evaluation of whole slide images, there are some important challenges to overcome. Tizhoosh et al. mentioned three key elements: interoperability, cost, and trust [260]. For a more in-depth general discussion on these factors, we refer to the original papers [260, 261]. Specifically in the context of

Table 13.6 Overview of methods of automated sTIL assessment in tumors. Adapted with permission from Amgad et al. [208]

Method	Approach	Stain	Notes
CNN [249]	Patch classification	H&E	*Strengths*: spatial information on sTIL. Molecular correlations *Limitations*: no distinction between sTIL and iTIL. No classification of individual TILs
FCN [258]	Semantic segmentation	H&E	*Strengths*: large sample size and regions. Delineation of tumor, stroma, and necrosis regions. *Limitations*: only detects dense TIL infiltrates. No classification of individual TILs.
Seeding + FCN [259]	Semantic segmentation + object detection	H&E	*Limitations*: heavy ground truth requirement
SVM	Object detection	H&E	*Strengths*: robust analysis and correlation with molecular TIL *Limitations*: individual labeled nuclei are limited. No distinction of TILs in different histologic regions
	Object detection + inferred TIL localization	H&E	*Strengths*: spatial localization and patterns. Robust. *Limitations*: individual labeled nuclei are limited. 1:1 correspondence clustering vs regions unclear
RG + MRF [250]	Object detection	H&E	*Strengths*: explainable model and modular pipeline. *Limitations*: no distinction between sTIL and iTIL. No classification of individual TILs
Watershed + SVM	Object detection	H&E	*Strengths*: explainable model. Robust. Spatial TIL clustering. *Limitations*: no distinction between sTIL and iTIL
Complex pipeline	Object detection + manual regions	IHC	*Strengths*: assessment of manual regions, including invasive margin.

TILs assessment, there are certainly trust challenges to overcome. These challenges are usually the reason for a high discordance rate between manual and automated assessment. Differences between both assessments can explain the discordance rate for the major part. Manual assessment is usually an estimation of the amount of mononuclear inflammatory cells in the stroma, while automated assessments predict a more accurate percentage. Moreover, in some tumor types (e.g., melanoma) different systems are used for categorization of the percentage of TILs, further explaining discordance. Another challenge in automated sTIL assessment is the characterization of the cells in the stroma. Not all of these cells are lymphocytes and not all lymphocytes have the same phenotype. Other cells that might be encountered are macrophages, fibroblasts, plasma cells, granulocytes, mast cells, etc. In normal circumstances, the AI-algorithm takes the size of the cell, the size of the nucleus and the area of the cytoplasm into consideration, but it is not aware that these measurements are highly dependent on the cut level through the cell, which might lead to a wrong identification of a cell. This is also an issue in manual assessment, although the human mind can easily consider this pitfall. Nonetheless, the prognostic value of sTIL is more robust when assessed with automated algorithms.

To have the most accurate result, the automated TILS assessment needs to follow the guidelines for scoring TILs as closely as possible. Therefore the efforts of the TILs-WG to standardize TILs assessment methodology from the very beginning are important to create a stable framework that contributes to development of reproducible algorithms. A good biomarker is considered analytically valid, reproducible, affordable, accessible, and clinically useful. It is in this context that Gonzalez-Ericsson et al. applied a risk management framework for the implementation of TILS next to PD-L1 assessment as immune-oncology biomarkers in daily practice and clinical trials. This application paves the pathway for further recommendations and guidelines regarding TILS scoring, improving reproducibility. The authors also mention the use of AI-based methods, but like any other biomarker computer-aided analysis should be analytically and clinically valid [165].

The predictive use of AI-based analysis of TILs is still somewhat controversial. To this date, no clinical trials concerning computer-aided TILs assessment exist, evaluating the efficacy of immune checkpoint inhibition. This is in contrast to the growing body of evidences illustrating that AI-assisted assessment of TILs is linked to prognosis. But of course, as AI is becoming more and more prominent, it is just a matter of time before the first trials with automated analysis of TILs counts will appear.

CNN convolutional neural networks, *sTIL* stromal tumor infiltrating lymphocytes, *iTIL* intratumoral infiltrating lymphocytes, *FCN* full convolutional neural network, *SVM* support vector machine, *RG* region growing, *MRF* Markov random field, *DL* deep learning

Concluding Remarks/Summary

The semiquantitative assessment of TILs in breast cancer (and in other tumors) is a well-defined histopathological parameter of which the assessment can easily be integrated

in the standard examination of biopsies and resection specimens by the (surgical) pathologist. It contributes valuable prognostic and predictive information that should be taken into account—together with other tumor characteristics—when discussing treatment options, especially in Her2-positive and TNBC. Over the past years, the International Immuno-Oncology Biomarkers Working Group (www.tilsinbreastcancer.org) has been involved in multiple studies to further characterize its value as a biomarker and it has also taken several educational initiatives and developed several tools to create awareness, to teach pathologists how to assess TILs according to a reproducible methodology and to integrate TILs assessment with other (new) biomarkers in immuno-oncology. Without any doubt, progressively TILs assessment will further enter clinical practice in the coming years. A structured, coordinated, and scientific approach—as the Immuno-Oncology Biomarkers Working Group tries to propagate and support—should aim to avoid the chaos PD-L1 testing has brought to this field.

Putting genomic/transcriptomic/proteomic information into a spatial context is considered as "the next step" in understanding cancer and establishing novel standards of personalized treatment [217]. Pathologists have been using spatial expression profiling using antibody- DNA-, RNA- and morphology-based methods for decades, but traditional IHC or in situ hybridization methods only allow the simultaneous assessment of a handful of markers in a single tissue slide, which are subsequently evaluated in a semiquantitative way [232]. Multiplex IHC represent the ideal bridge between non-spatial single cell technologies and the traditional monoplex approach of standard pathology providing useful insight about the spatial distribution of the diverse cellular components in a tumor mass. It is anticipated that using these types of approaches in well-designed early phase clinical trials will boost our knowledge about ICB therapies and improve personalized medicine [149, 154, 197, 233].

Furthermore, it is undebatable that computer-assisted TILs assessment can further characterize the tumor microenvironment beyond the capabilities of human minds. It is anticipated that AI-enabled methods will confirm more and more the prognostic and predictive value of TILs, therefore it seems logic to proceed with the implementation of these AI models in practice and in clinical trials. The robustness of these assessments will only increase when combined with the power of multiplexing immunohistochemistry for further characterization of the inflammatory infiltrate [208, 248].

As several AI models also investigate spatial information concerning sTIL, this spatial information can become part of the investigational subjects to further research the prognostic and predictive value of TILs. Not only distances between infiltrates or infiltrates and tumor cells can be considered, but also heterogeneity will also become more important [231, 248, 249].

Many current AI algorithms are based on image analysis, but in a next step additional clinical information and genomic data can be integrated in the analysis as well in order to create more robust models toward personalized and precision medicine. By integrating more and more data, these models are able to detect other prognostic or predictive patterns that are at first, invisible to human perception [208, 248].

References

1. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. Nat Rev Dis Primers. 2019;5:66.
2. Loibl S, Poortmans P, Morrow M, Denkert C, Curigliano G. Seminar Breast cancer. Lancet. 2021;397:1750–69. https://doi.org/10.1016/S0140-6736.
3. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, et al. Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nat Rev Clin Oncol. 2015;13(4):228–41.
4. Sistrunk WE, MacCarty WCMD. Life expectancy following radical amputation for carcinoma of the breast. Ann Surg. 1922;75(1):61–9.
5. Moore OS, Foote FW. The relatively favorable prognosis of medullary carcinoma of the breast. Cancer. 1949;2(4):635–42.
6. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
7. Vitale I, Shema E, Loi S, Galluzzi L. Dynamic heterogeneity of cancer cells Intratumoral heterogeneity in cancer progression and response to immunotherapy. Nat Med. 2022;14(2):280. https://doi.org/10.1038/s41591-021-01233-9.
8. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3:991–8.
9. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006;6:715–27.
10. Teng MWL, Galon J, Fridman WH, Smyth MJ. From mice to humans: developments in cancer immunoediting. J Clin Invest. 2015;125:3338–46.
11. Schmid P, Salgado R, Park YH, Muñoz-Couselo E, Kim SB, Sohn J, et al. Pembrolizumab plus chemotherapy as neoadjuvant treatment of high-risk, early-stage triple-negative breast cancer: results from the phase 1b open-label, multicohort KEYNOTE-173 study. Ann Oncol. 2020;31(5):569–81.
12. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, Janjigian YY, et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat Genet. 2019;51:202–6.
13. McGrail DJ, Pilié PG, Rashid NU, Voorwerk L, Slagter M, Kok M, et al. High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types. Ann Oncol. 2021;32(5):661–72.
14. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILS) in breast cancer: recommendations by an International TILS Working Group 2014. Ann Oncol. 2015;26:259–71.
15. Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, 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 invasi breast carcinoma and ductal carcinoma in situ, metastatic

tumor deposits and areas for further research. Adv Anat Pathol. 2017;24:235–51.
16. Kos Z, Roblin E, Kim RS, Michiels S, Gallas BD, Chen W, et al. Pitfalls in assessing stromal tumor infiltrating lymphocytes (sTILs) in breast cancer. npj Breast Cancer. 2020;6(1):17.
17. Rakha EA, El-Sayed ME, Lee AHS, Elston CW, Grainge MJ, Hodi Z, et al. Prognostic significance of nottingham histologic grade in invasive breast carcinoma. J Clin Oncol. 2008;26(19):3153–8.
18. WHO Classification of Tumours Editorial Board. Breast Tumours WHO Classification of Tumours, 5th Edition, Volume 2. oard WC of tumours editorial, editor. International Agency for Research on Cancer; 2019.
19. Monard S, Gorana T, Patrizia C, Andrea B, Silvana P, Natale C, et al. Lymphoid infiltration as a prognostic variable for early-onset breast carcinomas. Clin Cancer Res. 1997;3:817–9.
20. Guarneri V, Dieci MV, Bisagni G, Brandes AA, Frassoldati A, Cavanna L, et al. PIK3CA mutation in the shortHER randomized adjuvant trial for patients with early HER2þ breast cancer: Association with prognosis and integration with PAM50 subtype. Clin Cancer Res. 2020;26(22):5843–51.
21. Sasaki R, Horimoto Y, Yanai Y, Kurisaki-Arakawa A, Arakawa A, Nakai K, et al. Molecular characteristics of lymphocyte-predominant triple-negative breast cancer. Anticancer Res. 2021;41(4):2133–40.
22. Craven KE, Gökmen-Polar Y, Badve SS. CIBERSORT analysis of TCGA and METABRIC identifies subgroups with better outcomes in triple negative breast cancer. Sci Rep. 2021;11(1):4691.
23. Desmedt C, Salgado R, Fornili M, Pruneri G, van den Eynden G, Zoppoli G, et al. Immune infiltration in invasive lobular breast cancer. J Natl Cancer Inst. 2018;110(7):768–76.
24. Stanton SE, Adams S, Disis ML. Variation in the incidence and magnitude of tumor-infiltrating lymphocytes in breast cancer subtypes. JAMA Oncol. 2016;2(10):1354.
25. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19(1):40–50.
26. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol. 2013;31(7):860–7.
27. Denkert C, Loibl S, Noske A, Roller M, Müller BM, Komor M, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol. 2010;28(1):105–13.
28. Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, 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. In: Advances in anatomic pathology, vol. 24. Lippincott Williams and Wilkins; 2017. p. 235–51.
29. Rakha EA, Bennett RL, Coleman D, Pinder SE, Ellis IO. Review of the national external quality assessment (EQA) scheme for breast pathology in the UK. J Clin Pathol. 2017;70(1):51–7.
30. Morrow M, Schnitt SJ, Norton L. Current management of lesions associated with an increased risk of breast cancer. Nat Rev Clin Oncol. 2015;12(4):227–38.
31. Kim M, Chung YR, Kim HJ, Woo JW, Ahn S, Park SY. Immune microenvironment in ductal carcinoma in situ: a comparison with invasive carcinoma of the breast. Breast Cancer Res. 2020;22(1):32.
32. Chen X-Y, Yeong J, Thike AA, Bay BH, et al. Prognostic role of immune infiltrates in breast ductal carcinoma in situ. Breast Cancer Res Treat. 2019;177:17–27. https://doi.org/10.1007/s10549-019-05272-2.
33. Agahozo MC, van Bockstal MR, Groenendijk FH, van den Bosch TPP, Westenend PJ, van Deurzen CHM. Ductal carcinoma in situ of the breast: immune cell composition according to subtype. Mod Pathol. 2020;33(2):196–205.
34. Toss MS, Abidi A, Lesche D, Joseph C, Mahale S, Saunders H, et al. The prognostic significance of immune microenvironment in breast ductal carcinoma in situ. Br J Cancer. 2020;122(10):1496–506.
35. Gil Del Alcazar CR, Huh SJ, Ekram MB, Trinh A, Liu LL, Beca F, et al. Immune escape in breast cancer during in situ to invasive carcinoma transition. Cancer Discov. 2017;7(10):1098–115.
36. Hendry S, Pang JMB, Byrne DJ, Lakhani SR, Cummings MC, Campbell IG, et al. Relationship of the breast ductal carcinoma in situ immune microenvironment with clinicopathological and genetic features. Clin Cancer Res. 2017;23(17):5210–7.
37. Pruneri G, Lazzeroni M, Bagnardi V, Tiburzio GB, Rotmensz N, DeCensi A, 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):321–8.
38. Toss MS, Miligy I, Al-Kawaz A, Alsleem M, Khout H, Rida PC, et al. Prognostic significance of tumor-infiltrating lymphocytes in ductal carcinoma in situ of the breast. Mod Pathol. 2018;31(8):1226–36.
39. Dano H, Altinay S, Arnould L, Bletard N, Colpaert C, Dedeurwaerdere F, et al. Interobserver variability in upfront dichotomous histopathological assessment of ductal carcinoma in situ of the breast: the DCISion study. Mod Pathol. 2020;33(3):354–66.
40. Groen EJ, Hudecek J, Mulder L, van Seijen M, Almekinders MM, Alexov S, et al. Prognostic value of histopathological DCIS features in a large-scale international interrater reliability study. Breast Cancer Res Treat. 2020;183(3):759–70.
41. Cserni G, Sejben A. Grading ductal carcinoma in situ (DCIS) of the breast – what's wrong with it? Pathol Oncol Res. 2020;26:665–71.
42. Van Bockstal M, Lambein K, Smeets A, Slembrouck L, Neven P, Nevelsteen I, et al. Stromal characteristics are adequate prognosticators for recurrence risk in ductal carcinoma in situ of the breast. Eur J Surg Oncol. 2019;45(4):550–9.
43. Darvishian F, Ozerdem U, Adams S, Chun J, Pirraglia E, Kaplowitz E, et al. Tumor-infiltrating lymphocytes in a contemporary cohort of women with ductal carcinoma in situ (DCIS). Ann Surg Oncol. 2019;26(10):3337–43.
44. Thike AA, Chen X, Koh VCY, Binte md Nasir ND, JPS Y, Bay BH, et al. Higher densities of tumour-infiltrating lymphocytes and CD4+ T cells predict recurrence and progression of ductal carcinoma in situ of the breast. Histopathology. 2020;76(6):852–64.
45. Xu FF, Zheng SF, Xu C, Cai G, Wang SB, Qi WX, et al. Prognostic and predictive significance of tumor infiltrating lymphocytes for ductal carcinoma in situ. OncoImmunology. 2021;10(1):1875637.
46. Farolfi A, Petracci E, Serra L, Ravaioli A, Bravaccini S, Ravaioli S, et al. Tumor-infiltrating lymphocytes (TILs) and risk of a second breast event after a ductal carcinoma in situ. Front Oncol. 2020;10:1486.
47. Ades F, Zardavas D, Bozovic-Spasojevic I, Pugliano L, Fumagalli D, De Azambuja E, et al. Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. J Clin Oncol. 2014;32:2794–803.
48. Pondé NF, Zardavas D, Piccart M. Progress in adjuvant systemic therapy for breast cancer. Nat Rev Clin Oncol. 2019;16:27–44.
49. Sparano JA, Gray RJ, Ravdin PM, Makower DF, Pritchard KI, Albain KS, et al. Clinical and genomic risk to guide the

use of adjuvant therapy for breast cancer. N Engl J Med. 2019;380(25):2395–405.
50. Piccart M, van 't Veer LJ, Poncet C, Lopes Cardozo JMN, Delaloge S, Pierga JY, et al. 70-gene signature as an aid for treatment decisions in early breast cancer: updated results of the phase 3 randomised MINDACT trial with an exploratory analysis by age. Lancet Oncol. 2021;22(4):476–88.
51. Laws A, Garrido-Castro AC, Poorvu PD, Winer EP, Mittendorf EA, King TA. Utility of the 21-gene recurrence score in node-positive breast cancer. Oncology. 2021;35:77–84.
52. Acs G, Esposito NN, Kiluk J, Loftus L, Laronga C. A mitotically active, cellular tumor stroma and/or inflammatory cells associated with tumor cells may contribute to intermediate or high Oncotype DX Recurrence Scores in low-grade invasive breast carcinomas. Mod Pathol. 2012;25(4):556–66.
53. Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G, et al. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Cancer Res. 2008;14(16):5158–65.
54. Krishnamurti U, Wetherilt CS, Yang J, Peng L, Li X. Tumor-infiltrating lymphocytes are significantly associated with better overall survival and disease-free survival in triple-negative but not estrogen receptor–positive breast cancers. Hum Pathol. 2017;64:7–12.
55. Ahn SG, Cha YJ, Bae SJ, Yoon C, Lee HW, Jeong J. Comparisons of tumor-infiltrating lymphocyte levels and the 21-gene recurrence score in ER-positive/HER2-negative breast cancer. BMC Cancer. 2018;18(1):320.
56. Kolberg-Liedtke C, Oleg G, Fred H, Friedrich F, Hans K, Michael C, et al. Association of TILs with clinical parameters, Recurrence Score® results, and prognosis in patients with early HER2-negative breast cancer (BC) - a translational analysis of the prospective WSG PlanB trial. Breast Cancer Res. 2020;22(1):47.
57. Nagalla S, Chou JW, Willingham MC, Ruiz J, Vaughn JP, Dubey P, et al. Interactions between immunity, proliferation and molecular subtype in breast cancer prognosis. Genome Biol. 2013;14(4):R34.
58. Seelige R, Searles S, Jack ·, Bui D. Mechanisms regulating immune surveillance of cellular stress in cancer. Cell Mol Life Sci. 2018;75:225–40.
59. López-Soto A, Gonzalez S, López-Larrea C, Kroemer G. Immunosurveillance of malignant cells with complex karyotypes. Trends Cell Biol. 2017;27:880–4.
60. Smid M, Rodríguez-González FG, Sieuwerts AM, Salgado R, Prager-Van Der Smissen WJC, van der Vlugt-Daane M, et al. Breast cancer genome and transcriptome integration implicates specific mutational signatures with immune cell infiltration. Nat Commun. 2016;7:12910.
61. Solinas C, Marcoux D, Garaud S, Vitória JR, van den Eynden G, de Wind A, et al. BRCA gene mutations do not shape the extent and organization of tumor infiltrating lymphocytes in triple negative breast cancer. Cancer Lett. 2019;450:88–97.
62. Haricharan S, Bainbridge MN, Scheet P, Brown PH. Somatic mutation load of estrogen receptor-positive breast tumors predicts overall survival: An analysis of genome sequence data. Breast Cancer Res Treat. 2014;146(1):211–20.
63. Davies H, Morganella S, Purdie CA, Jang SJ, Borgen E, Russnes H, et al. Whole-genome sequencing reveals breast cancers with mismatch repair deficiency. Cancer Res. 2017;77(18):4755–62.
64. Denkert C, Von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. J Clin Oncol. 2015;33(9):983–91.
65. Heng YJ, Lester SC, Tse GMK, Factor RE, Allison KH, Collins LC, et al. The molecular basis of breast cancer pathological phenotypes. J Pathol. 2017;241(3):375–91.
66. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol. 2014;25(8):1544–50.
67. Dieci MV, Mathieu MC, Guarneri V, Conte P, Delaloge S, Andre F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. Ann Oncol. 2015;26(8):1698–704.
68. Fujimoto Y, Watanabe T, Hida AI, Higuchi T, Miyagawa Y, Ozawa H, et al. Prognostic significance of tumor-infiltrating lymphocytes may differ depending on Ki67 expression levels in estrogen receptor-positive/HER2-negative operated breast cancers. Breast Cancer. 2019;26(6):738–47.
69. Criscitiello C, Vingiani A, Maisonneuve P, Viale G, Viale G, Curigliano G. Tumor-infiltrating lymphocytes (TILs) in ER+/HER2− breast cancer. Breast Cancer Res Treat. 2020;183(2):347–54.
70. Hua GZ, Xin LC, Liu M, Yuan JJ. Predictive and prognostic role of tumour-infiltrating lymphocytes in breast cancer patients with different molecular subtypes: a meta-analysis. BMC Cancer. 2020;20(1):1150.
71. Deman F, Punie K, Laenen A, Neven P, Oldenburger E, Smeets A, et al. Assessment of stromal tumor infiltrating lymphocytes and immunohistochemical features in invasive micropapillary breast carcinoma with long-term outcomes. Breast Cancer Res Treat. 2020;184(3):985–98.
72. Richard F, Majjaj S, Venet D, Rothé F, Pingitore J, Boeckx B, et al. Characterization of stromal tumor-infiltrating lymphocytes and genomic alterations in metastatic lobular breast cancer. Clin Cancer Res. 2020;26(23):6254–65.
73. Guo X, Chen L, Lang R, Fan Y, Zhang X, Fu L. Invasive micropapillary carcinoma of the breast: association of pathologic features with lymph node metastasis. Am J Clin Pathol. 2006;126(5):740–6.
74. Gucalp A, Traina TA, Eisner JR, Parker JS, Selitsky SR, Park BH, et al. Male breast cancer: a disease distinct from female breast cancer. Breast Cancer Res Treat. 2019;173:37–48.
75. Vermeulen MA, Slaets L, Cardoso F, Giordano SH, Tryfonidis K, van Diest PJ, et al. Pathological characterisation of male breast cancer: results of the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program. Eur J Cancer. 2017;82:219–27.
76. Ali HR, Provenzano E, Dawson S-J, Blows FM, Liu B, Shah M, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients. Ann Oncol. 2014;25:1536–43.
77. Mahmoud SMA, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AHS, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol. 2011;29(15):1949–55.
78. Dieci MV, Miglietta F, Guarneri V. Immune infiltrates in breast cancer: recent updates and clinical implications. Cells. 2021;10:223.
79. Scheel AH, Penault-Llorca F, Hanna W, Baretton G, Middel P, Burchhardt J, et al. Physical basis of the "magnification rule" for standardized Immunohistochemical scoring of HER2 in breast and gastric cancer. Diagn Pathol. 2018;13(1)
80. Wolff AC, McShane LM, Hammond MEH, Allison KH, Fitzgibbons P, Press MF, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. Arch Pathol Lab Med. 2018;142:1364–82.
81. Goutsouliak K, Veeraraghavan J, Sethunath V, Angelis C, Kent Osborne C, Rimawi MF, et al. Towards personalized treatment for early stage HER2-positive breast cancer. Nat Rev Clin Oncol. 2020;17(4):233–50.

82. Rimawi MF, Schiff R, Osborne CK. Targeting HER2 for the treatment of breast cancer. Annu Rev Med. 2015;66:111–28.
83. Brandão M, Caparica R, Malorni L, Prat A, Carey LA, Piccart M. What is the real impact of estrogen receptor status on the prognosis and treatment of HER2-positive early breast cancer? Clin Cancer Res. 2020;26:2783–8.
84. Marchiò C, Annaratone L, Marques A, Casorzo L, Berrino E, Sapino A. Evolving concepts in HER2 evaluation in breast cancer: heterogeneity, HER2-low carcinomas and beyond. Semin Cancer Biol. 2021;72:123–35.
85. Schettini F, Chic N, Brasó-Maristany F, Paré L, Pascual T, Conte B, et al. Clinical, pathological, and PAM50 gene expression features of HER2-low breast cancer. NPJ Breast Cancer. 2021;7(1):1.
86. Salgado R, Denkert C, Campbell C, Savas P, Nuciforo P, Aura C, et al. Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early-stage breast cancer treated with lapatinib and trastuzumab: A secondary analysis of the NeoALTTO trial. JAMA Oncol. 2015;1(4):448–55.
87. Rody A, Holtrich U, Pusztai L, Liedtke C, Gaetje R, Ruckhaeberle E, et al. T-cell metagene predicts a favorable prognosis in estrogen receptor-negative and HER2-positive breast cancers. Breast Cancer Res. 2009;11(2):R15.
88. Perez EA, Ballman KV, Tenner KS, Thompson EA, Badve SS, Bailey H, et al. Association of stromal tumor-infiltrating lymphocytes with recurrence-free survival in the n9831 adjuvant trial in patients with early-stage HER2-positive breast cancer. JAMA Oncol. 2016;2(1):56–64.
89. Kim RS, Song N, Gavin PG, Salgado R, Bandos H, Kos Z, et al. Stromal tumor-infiltrating lymphocytes in NRG oncology/NSABP B-31 adjuvant trial for early-stage HER2-positive breast cancer. J Natl Cancer Inst. 2019;111(8):867–71.
90. Pogue-Geile KL, Song N, Serie DJ, Wang Y, Gavin PG, Kim RS, et al. Validation of the NSABP/NRG Oncology 8-gene trastuzumab-benefit signature in alliance/NCCTG N9831. JNCI Cancer Spectr. 2020;4(5):pkaa058.
91. Dieci MV, Conte P, Bisagni G, Brandes AA, Frassoldati A, Cavanna L, 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):418–23.
92. Prat A, Guarneri V, Paré L, Griguolo G, Pascual T, Dieci MV, et al. A multivariable prognostic score to guide systemic therapy in early-stage HER2-positive breast cancer: a retrospective study with an external evaluation. Lancet Oncol. 2020;21(11):1455–64.
93. He L, Wang Y, Wu Q, Song Y, Ma X, Zhang B, et al. Association between levels of tumor-infiltrating lymphocytes in different subtypes of primary breast tumors and prognostic outcomes: a meta-analysis. BMC Women's Health. 2020;20(1):194.
94. Desmedt C, Zoppoli G, Sotiriou C, Salgado R. Transcriptomic and genomic features of invasive lobular breast cancer. Semin Cancer Biol. 2017;44:98–105.
95. Jongen L, Floris G, Boeckx B, Smeets D, Lambrechts D, van der Borght S, et al. Identification, clinical-pathological characteristics and treatment outcomes of patients with metastatic breast cancer and somatic human epidermal growth factor receptor 2 (ERBB2) mutations. Breast Cancer Res Treat. 2019;174(1):55–63.
96. Geyer FC, Pareja F, Weigelt B, Rakha E, Ellis IO, Schnitt SJ, et al. The spectrum of triple-negative breast disease: high- and low-grade lesions. Am J Pathol. 2017;187:2139–51.
97. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Investig. 2011;121(7):2750–67.
98. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua SAW, et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. Clin Cancer Res. 2015;21(7):1688–98.
99. Turner NC, Reis-Filho JS. Tackling the diversity of Triple-negative breast cancer. Clin Cancer Res. 2013;19(23):6380–8.
100. Bertucci F, Finetti P, Cervera N, Charafe-Jauffret E, Mamessier E, Adélaïdev JA, et al. Gene expression profiling shows medullary breast cancer is a subgroup of basal breast cancers. Cancer Res. 2006;66(9):4636–80.
101. Lehmann BD, Jovanović B, Chen X, Estrada MV, Johnson KN, Shyr Y, et al. Refinement of triple-negative breast cancer molecular subtypes: implications for neoadjuvant chemotherapy selection. 2016. Available from: http://cancer
102. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol. 2014;32:2959–66.
103. Pruneri G, Gray KP, Vingiani A, Viale G, Curigliano G, Criscitiello C, et al. Tumor-infiltrating lymphocytes (TILs) are a powerful prognostic marker in patients with triple-negative breast cancer enrolled in the IBCSG phase III randomized clinical trial 22-00. Breast Cancer Res Treat. 2016;158:323–31.
104. Loi S, Drubay D, Adams S, Pruneri G, Francis PA, Lacroix-Triki M, et al. Tumor-infiltrating lymphocytes and prognosis: a pooled individual patient analysis of early-stage triple-negative breast cancers. J Clin Oncol. 2019;37:559–69.
105. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. The Lancet. 2014;384(9938):164–72.
106. Provenzano E, Bossuyt V, Viale G, Cameron D, Badve S, Denkert C, et al. Standardization of pathologic evaluation and reporting of postneoadjuvant specimens in clinical trials of breast cancer: recommendations from an international working group. 2015. Available from: www.modernpathology.org
107. Symmans WF, Peintinger F, Hatzis C, Rajan R, Kuerer H, Valero V, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. J Clin Oncol. 2007;25(28):4414–22.
108. Ogston KN, Miller ID, Payne S, Hutcheon AW, Sarkar TK, Smith I, et al. A new histological grading system to assess response of breast cancers to primary chemotherapy: prognostic significance and survival. Breast. 2003;12(5):320–7.
109. Wein L, Luen SJ, Savas P, Salgado R, Loi S. Checkpoint blockade in the treatment of breast cancer: current status and future directions. 2018. https://doi.org/10.1038/s41416-018-0126-6.
110. von Minckwitz G, Untch M, Blohmer JU, Costa SD, Eidtmann H, Fasching PA, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Oncol. 2012;30(15):1796–804.
111. Schneeweiss A, Möbus V, Tesch H, Hanusch C, Denkert C, Lübbe K, et al. Intense dose-dense epirubicin, paclitaxel, cyclophosphamide versus weekly paclitaxel, liposomal doxorubicin (plus carboplatin in triple-negative breast cancer) for neoadjuvant treatment of high-risk early breast cancer (GeparOcto—GBG 84): a randomised phase III trial. Eur J Cancer. 2019;106:181–92.
112. Issa-Nummer Y, Darb-Esfahani S, Loibl S, Kunz G, Nekljudova V, Schrader I, et al. Prospective validation of immunological infiltrate for prediction of response to neoadjuvant chemotherapy in HER2-negative breast cancer – a substudy of the neoadjuvant GeparQuinto trial. PLoS One. 2013;8(12):e79775. https://doi.org/10.1371/journal.pone.0079775.
113. O'Loughlin M, Andreu X, Bianchi S, Chemielik E, Cordoba A, Cserni G, et al. Reproducibility and predictive value of scoring stromal tumour infiltrating lymphocytes in triple-negative breast

cancer: a multi-institutional study. Breast Cancer Res Treat. 2021;171(1):1–9.
114. Denkert C, Wienert S, Poterie A, Loibl S, Budczies J, Badve S, et al. Standardized evaluation of tumor-infiltrating lymphocytes in breast cancer: results of the ring studies of the international immuno-oncology biomarker working group. Mod Pathol. 2016;29(10):1155–64.
115. Asano Y, Kashiwagi S, Goto W, Takada K, Takahashi K, Hatano T, et al. Prediction of treatment response to neoadjuvant chemotherapy in breast cancer by subtype using tumor-infiltrating lymphocytes. Anticancer Res. 2018;38(4):2311–21.
116. Jongen L, Floris G, Wildiers H, Claessens F, Richard F, Laenen A, et al. Tumor characteristics and outcome by androgen receptor expression in triple-negative breast cancer patients treated with neo-adjuvant chemotherapy. Br Cancer Res Treat. 2019;176:699–708. https://doi.org/10.1007/s10549-019-05252-6.
117. Hamy AS, Bonsang-Kitzis H, de Croze D, Laas E, Darrigues L, Topciu L, et al. Interaction between molecular subtypes and stromal immune infiltration before and after treatment in breast cancer patients treated with neoadjuvant chemotherapy. Clin Cancer Res. 2019;25(22):6731–41.
118. Gao G, Wang Z, Qu X, Zhang Z. Prognostic value of tumor-infiltrating lymphocytes in patients with triple-negative breast cancer: a systematic review and meta-analysis. BMC Cancer. 2020;20(1):179.
119. Luen SJ, Salgado R, Loi S. Residual disease and immune infiltration as a new surrogate endpoint for TNBC post neoadjuvant chemotherapy. Oncotarget. 2019;10:4612–4.
120. Demaria S, Volm MD, Shapiro RL, Yee HY, Oratz R, Formenti SC, et al. Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. Clin Cancer Res. 2001;7(10):3025–30.
121. Dieci MV, Criscitiello C, Goubar A, Viale G, Conte P, Guarneri V, et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: a retrospective multicenter study. Ann Oncol. 2014;25(3):611–8.
122. Loi S, Dushyanthen S, Beavis PA, Salgado R, Denkert C, Savas P, et al. RAS/MAPK activation is associated with reduced tumor-infiltrating lymphocytes in triple-negative breast cancer: Therapeutic cooperation between MEK and PD-1/PD-L1 immune checkpoint inhibitors. Clin Cancer Res. 2016;22(6):1499–509.
123. Luen SJ, Salgado R, Dieci MV, Vingiani A, Curigliano G, Gould RE, et al. Prognostic implications of residual disease tumor-infiltrating lymphocytes and residual cancer burden in triple-negative breast cancer patients after neoadjuvant chemotherapy. Ann Oncol. 2019;30(2):236–42.
124. Ali HR, Dariush A, Thomas J, Provenzano E, Dunn J, Hiller L, et al. Lymphocyte density determined by computational pathology validated as a predictor of response to neoadjuvant chemotherapy in breast cancer: secondary analysis of the ARTemis trial. Ann Oncol. 2017;28(8):1832–5.
125. Ali HR, Dariush A, Provenzano E, Bardwell H, Abraham JE, Iddawela M, et al. Computational pathology of pre-treatment biopsies identifies lymphocyte density as a predictor of response to neoadjuvant chemotherapy in breast cancer. Breast Cancer Res. 2016;18(1):21.
126. Dieci MV, Frassoldati A, Generali D, Bisagni G, Piacentini F, Cavanna L, et al. Tumor-infiltrating lymphocytes and molecular response after neoadjuvant therapy for HR+/HER2− breast cancer: results from two prospective trials. Br Cancer Res Treat. 2017;163(2):295–302.
127. Griguolo G, Dieci MV, Paré L, Miglietta F, Generali DG, Frassoldati A, et al. Immune microenvironment and intrinsic subtyping in hormone receptor-positive/HER2-negative breast cancer. NPJ Br Cancer. 2021;7(1):1–5. https://doi.org/10.1038/s41523-021-00223-x.
128. Badr NM, Spooner D, Steven J, Stevens A, Shaaban AM. Morphological and molecular changes following neoadjuvant endocrine therapy of ER positive breast cancer: implications for clinical practice. Histopathology. 2021;79(1):47–56.
129. Skriver SK, Jensen MB, Knoop AS, Ejlertsen B, Laenkholm AV. Tumour-infiltrating lymphocytes and response to neoadjuvant letrozole in patients with early oestrogen receptor-positive breast cancer: analysis from a nationwide phase II DBCG trial. Breast Cancer Res. 2020;22(1):46.
130. Dunbier AK, Ghazoui Z, Anderson H, Salter J, Nerurkar A, Osin P, et al. Molecular profiling of aromatase inhibitor-treated postmenopausal breast tumors identifies immune-related correlates of resistance. Clin Cancer Res. 2013;19(10):2775–86.
131. Liang X, Briaux A, Becette V, Benoist C, Boulai A, Chemlali W, et al. Molecular profiling of hormone receptor-positive, HER2-negative breast cancers from patients treated with neoadjuvant endocrine therapy in the CARMINA 02 trial (UCBG-0609). J Hematol Oncol. 2018;11(1):124.
132. Chan MSM, Wang L, Felizola SJA, Ueno T, Toi M, Loo W, et al. Changes of tumor infiltrating lymphocyte subtypes before and after neoadjuvant endocrine therapy in estrogen receptor-positive breast cancer patients - an immunohistochemical study of cd8+ and foxp3+ using double immunostaining with correlation to the pathobiological response of the patients. Int J Biol Markers. 2012;27(4):e295–304.
133. Llombart-Cussac A, Cortés J, Paré L, Galván P, Bermejo B, Martínez N, et al. HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial. Lancet Oncol. 2017;18(4):545–54.
134. Nuciforo P, Pascual T, Cortés J, Llombart-Cussac A, Fasani R, Paré L, et al. A predictive model of pathologic response based on tumor cellularity and tumor-infiltrating lymphocytes (CelTIL) in HER2-positive breast cancer treated with chemo-free dual HER2 blockade. Ann Oncol. 2018;29(1):170–7.
135. Chic N, Luen SJ, Nuciforo P, Salgado R, Fumagalli D, Hilbers F, et al. Tumor cellularity and infiltrating lymphocytes (CelTIL) as a survival surrogate in HER2-positive breast cancer. J Natl Cancer Inst. 2022;114(3):467–70.
136. Desmedt C, Fornili M, Clatot F, Demicheli R, de Bortoli D, di Leo A, et al. Differential benefit of adjuvant docetaxel-based chemotherapy in patients with early breast cancer according to baseline body mass index. J Clin Oncol. 2020;38(25):2883–91.
137. Floris G, Richard F, Hamy A-S, Jongen L, Wildiers H, Ardui J, et al. Body mass index and tumor-infiltrating lymphocytes in triple-negative breast cancer. J Natl Cancer Inst. 2021;113(2):146–53.
138. Murphy WJ, Longo DL. The surprisingly positive association between obesity and cancer immunotherapy efficacy. J Am Med Assoc. 2019;321:1247–8.
139. Cardoso F, Paluch-Shimon S, Senkus E, Curigliano G, Aapro MS, André F, et al. 5th ESO-ESMO international consensus guidelines for advanced breast cancer (ABC 5). Ann Oncol. 2020;31(12):1623–49.
140. Wein L, Savas P, Luen SJ, Virassamy B, Salgado R, Loi S. Clinical validity and utility of tumor-infiltrating lymphocytes in routine clinical practice for breast cancer patients: current and future directions. Front Oncol. 2017;7:156.
141. Savas P, Virassamy B, Ye C, Salim A, Mintoff CP, Caramia F, 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):986–93.
142. Loi S, Giobbie-Hurder A, Gombos A, Bachelot T, Hui R, Curigliano G, 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):371–82.
143. Adams S, Schmid P, Rugo HS, Winer EP, Loirat D, Awada A, et al. Pembrolizumab monotherapy for previously treated metastatic triple-negative breast cancer: cohort A of the phase II KEYNOTE-086 study. Ann Oncol. 2019;30(3):397–404.
144. Ogiya R, Niikura N, Kumaki N, Bianchini G, Kitano S, Iwamoto T, et al. Comparison of tumor-infiltrating lymphocytes between primary and metastatic tumors in breast cancer patients. Cancer Sci. 2016;107(12):1730–5.
145. Cimino-Mathews A, Ye X, Meeker A, Argani P, Emens LA. Metastatic triple-negative breast cancers at first relapse have fewer tumor-infiltrating lymphocytes than their matched primary breast tumors: a pilot study. Hum Pathol. 2013;44(10):2055–63.
146. Ogiya R, Niikura N, Kumaki N, Yasojima H, Iwasa T, Kanbayashi C, et al. Comparison of immune microenvironments between primary tumors and brain metastases in patients with breast cancer. Oncotarget. 2017;8(61):103671–81.
147. Vermeulen PB, Bohlok A, Leduc S, Richard F, Botzenhart L, Ignatiadis M, et al. Abstract P3-01-13: Association between the histopathological growth patterns (HGP) of liver metastases (LM) and survival after hepatic surgery in patients with oligometastatic breast cancer (BC). In: Cancer Research. American Association for Cancer Research (AACR); 2020. p. P3-01-13-P3-01–13. Available from: https://cancerres.aacrjournals.org/content/80/4_Supplement/P3-01-13
148. van Dam PJ, van der Stok EP, Teuwen LA, van den Eynden GG, Illemann M, Frentzas S, et al. International consensus guidelines for scoring the histopathological growth patterns of liver metastasis. Br J Cancer. 2017;117:1427–41.
149. Sambade MJ, Prince G, Deal AM, Trembath D, McKee M, Garrett A, et al. Examination and prognostic implications of the unique microenvironment of breast cancer brain metastases. Brest Cancer Res Treat. 2019;176(2):321–8.
150. Szekely B, Bossuyt V, Li X, Wali VB, Patwardhan GA, Frederick C, et al. Immunological differences between primary and metastatic breast cancer. Ann Oncol. 2018;29(11):2232–9.
151. Dieci MV, Tsvetkova V, Orvieto E, Piacentini F, Ficarra G, Griguolo G, et al. Immune characterization of breast cancer metastases: Prognostic implications. Breast Cancer Res. 2018;20(1):62.
152. Luen SJ, Salgado R, Fox S, Savas P, Eng-Wong J, Clark E, 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.
153. Iacobuzio-Donahue CA, Michael C, Baez P, Kappagantula R, Hooper JE, Hollman TJ. Cancer biology as revealed by the research autopsy. Nat Rev Cancer. 2019;19(12):686–97.
154. Dankner M, Issa-Chergui B, Bouganim N. Post-mortem tissue donation programs as platforms to accelerate cancer research. J Pathol Clin Res. 2020;6(3):163–70.
155. Doroshow DB, Bhalla S, Beasley MB, Sholl LM, Kerr KM, Gnjatic S, et al. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. Nat Rev Clin Oncol. 2021;18(6):345–62.
156. Paver EC, Cooper WA, Colebatch AJ, Ferguson PM, Hill SK, Lum T, et al. Programmed death ligand-1 (PD-L1) as a predictive marker for immunotherapy in solid tumours: a guide to immunohistochemistry implementation and interpretation. Pathology. 2021;53:141–56.
157. Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, et al. 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.
158. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med. 2018;379(22):2108–21.
159. Cortes J, Cescon DW, Rugo HS, Nowecki Z, Im SA, Yusof MM, et al. Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. The Lancet. 2020;396(10265):1817–28.
160. Miles DW, Gligorov J, André F, Cameron D, Schneeweiss A, Barrios CH, et al. LBA15 Primary results from IMpassion131, a double-blind placebo-controlled randomised phase III trial of first-line paclitaxel (PAC) ± atezolizumab (atezo) for unresectable locally advanced/metastatic triple-negative breast cancer (mTNBC). Ann Oncol. 2020;31:S1147–8.
161. Lu S, Stein JE, Rimm DL, Wang DW, Bell JM, Johnson DB, et al. Comparison of biomarker modalities for predicting response to PD-1/PD-L1 checkpoint blockade: a systematic review and meta-analysis. JAMA Oncol. 2019;5:1195–204.
162. Schmid P, Cortes J, Pusztai L, McArthur H, Kümmel S, Bergh J, et al. Pembrolizumab for early triple-negative breast cancer. N Engl J Med. 2020;382(9):810–21.
163. Mittendorf EA, Zhang H, Barrios CH, Saji S, Jung KH, Hegg R, et al. Neoadjuvant atezolizumab in combination with sequential nab-paclitaxel and anthracycline-based chemotherapy versus placebo and chemotherapy in patients with early-stage triple-negative breast cancer (IMpassion031): a randomised, double-blind, phase 3 trial. Lancet. 2020;396(10257):1090–100.
164. Rugo H, Loi S, Adams S, Schmid P, Schneeweiss A, Barrios CH, et al. PD1-07. Exploratory analytical harmonization of PD-L1 immunohistochemistry assays in advanced triple-negative breast cancer: a retrospective substudy of IMpassion130. 42nd Annual CTRC-AACR San Antonio Breast Cancer Symposium, San Antonio, TX, December 10–14, 2019.
165. Gonzalez-Ericsson PI, Stovgaard ES, Sua LF, Reisenbichler E, Kos Z, Carter JM, 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;250:667–84.
166. Roche H-L (2015) A study of atezolizumab in combination with nab-paclitaxel compared with placebo with nab-paclitaxel for participants with previously untreated metastatic triple-negative breast cancer (IMpassion130), https://ClinicalTrials.gov/show/NCT02425891
167. Schmid P, et al. 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.
168. Rugo HS, Loi S, Adams S, Schmid P, Schneeweiss A, Barrios CH, et al. LBA20: performance of PD-L1 immunohistochemistry (IHC) assays in unresectable locally advanced or metastatic triple-negative breastcancer (mTNBC): post-hoc analysis of IMpassion130. Ann Oncol. 2019;30(S5):v858–9.
169. A study of atezolizumab and paclitaxel versus placebo and paclitaxel in participants with previously untreated locally advanced or metastatic triple negative breast cancer (TNBC), https://ClinicalTrials.gov/show/NCT03125902
170. Miles DW, Gligorov J, André F, Cameron D, Schneeweiss A, Barrios CH, et al. LBA15 - primary results from IMpassion131, a double-blind placebo-controlled randomised phase III trial of first-line paclitaxel (PAC) ± atezolizumab (atezo) for unresectable locally advanced/metastatic triple-negative breast cancer (mTNBC). Ann Oncol. 2020;31(suppl_4):S1142–215. https://doi.org/10.1016/annonc/annonc325.

171. A study to investigate atezolizumab and chemotherapy compared with placebo and chemotherapy in the neoadjuvant setting in participants with early stage triple negative breast cancer, https://ClinicalTrials.gov/show/NCT03197935
172. Study of single agent pembrolizumab (MK-3475) versus single agent chemotherapy for metastatic triple negative breast cancer (MK-3475-119/KEYNOTE-119), https://ClinicalTrials.gov/show/NCT02555657
173. Winer EP, Lipatov O, Im S-A, Goncalves A, Muñoz-Couselo E, Lee KS, et al. Pembrolizumab versus investigator-choice chemotherapy for metastatic triple-negative breast cancer (KEYNOTE-119): a randomised, open-label, phase 3 trial. Lancet Oncol. 2021;22(4):499–511.
174. Study of pembrolizumab (MK-3475) plus chemotherapy vs. placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple negative breast cancer (MK-3475-355/KEYNOTE-355), https://ClinicalTrials.gov/show/NCT02819518
175. Study of pembrolizumab (MK-3475) plus chemotherapy vs placebo plus chemotherapy as neoadjuvant therapy and pembrolizumab vs placebo as adjuvant therapy in participants with triple negative breast cancer (TNBC) (MK-3475-522/KEYNOTE-522), https://ClinicalTrials.gov/show/NCT03036488
176. Roche H-L (2019) A study of atezolizumab plus nab-paclitaxel in the treatment of unresectable locally advanced or metastatic PD-L1-positive triple-negative breast cancer, https://ClinicalTrials.gov/show/NCT04148911.
177. Inc NF et al. (2017) Clinical trial of neoadjuvant chemotherapy with atezolizumab or placebo in patients with triple-negative breast cancer followed after surgery by atezolizumab or placebo, https://ClinicalTrials.gov/show/NCT03281954.
178. Sharp M, Corp D (2019) Study of olaparib plus pembrolizumab versus chemotherapy plus pembrolizumab after induction with first-line chemotherapy plus pembrolizumab in triple negative breast cancer (TNBC) (MK-7339-009/KEYLYNK-009), https://ClinicalTrials.gov/show/NCT04191135
179. Roche H-L (2018) A study of the efficacy and safety of atezolizumab plus chemotherapy for patients with early relapsing recurrent triple-negative breast cancer, https://ClinicalTrials.gov/show/NCT03371017
180. Roche H-L, et al (2018) A study comparing atezolizumab (anti PD-L1 antibody) in combination with adjuvant anthracycline/taxane-based chemotherapy versus chemotherapy alone in patients with operable triple-negative breast cancer, https://ClinicalTrials.gov/show/NCT03498716
181. Michelangelo F (2016) Neoadjuvant therapy in TRIPle negative breast cancer with antiPDL1, https://ClinicalTrials.gov/show/NCT02620280
182. Jiangsu HengRui Medicine Co., L. (2020) A phase 3 study comparing carelizumab plus nab-paclitaxel and apatinib, carelizumab plus nab-paclitaxel, and nab-paclitaxel in patients with advanced triple negative breast cancer, https://ClinicalTrials.gov/show/NCT04335006
183. Jiangsu HengRui Medicine Co., L. (2020) A study of camrelizumab plus chemotherapy vs placebo plus chemotherapy as neoadjuvant therapy in participants with triple negative breast cancer (TNBC), https://ClinicalTrials.gov/show/NCT04613674
184. Shanghai Junshi Bioscience Co., L. (2018). Toripalimab in combination with nab-paclitaxel for patients with metastatic or recurrent triple-negative breast cancer (TNBC) with or without systemic treatment, https://ClinicalTrials.gov/show/NCT04085276
185. Roche H-L, Chugai Pharmaceutical (2019) A study to evaluate the efficacy and safety of atezolizumab or placebo in combination with neoadjuvant doxorubicin + cyclophosphamide followed by paclitaxel + trastuzumab + pertuzumab in early her2-positive breast cancer, https://ClinicalTrials.gov/show/NCT03726879
186. Roche H-L (2021) A study of trastuzumab emtansine in combination with atezolizumab or placebo as a treatment for participants with human epidermal growth factor 2 (HER2)-positive and programmed death-ligand 1 (PD-L1)-positive locally advanced (LABC) or metastatic breast cancer (MBC), https://ClinicalTrials.gov/show/NCT04740918
187. Japanese Foundation for Cancer Research and Chugai Pharmaceutical (2021) A phase III study of bevacizumab and paclitaxel in combination with atezolizumab as a treatment for locally advanced unresectable or metastatic hormone receptor-positive HER2 negative breast cancer, https://ClinicalTrials.gov/show/NCT04732598
188. Sharp M, Dohme Corp (2018). Study of pembrolizumab (MK-3475) versus placebo in combination with neoadjuvant chemotherapy & adjuvant endocrine therapy in the treatment of early-stage estrogen receptor-positive, human epidermal growth factor receptor 2-negative (ER+/HER2-) breast cancer (MK-3475-756/KEYNOTE-756), https://ClinicalTrials.gov/show/NCT03725059
189. Tannock IF. Have investigators forgotten how to write? Ann Oncol. 2021 Apr;32(4):437–8. https://doi.org/10.1016/j.annonc.2020.12.017.
190. Fundytus A, Booth CM, Tannock IF. How low can you go? PD-L1 expression as a biomarker in trials of cancer immunotherapy. Ann Oncol. 2021;S0923-7534(21):01118–2. https://doi.org/10.1016/j.annonc.2021.03.208.
191. Salgado R, Bellizzi AM, Rimm D, Bartlett JM, Nielsen T, Holger M, et al. How current assay approval policies are leading to unintended imprecision medicine. Ann Oncol. 2021 Apr;32(4):437–8. https://doi.org/10.1016/j.annonc.2020.12.017.
192. Ahmed FS, Gaule P, McGuire J, Patel K, Blenman K, Pusztai L, et al. PD-L1 Protein expression on both tumor cells and macrophages are associated with response to neoadjuvant durvalumab with chemotherapy in triple-negative breast cancer. Clin Cancer Res. 2020;26(20):5456–61.
193. Tang H, Wang Y, Chlewicki LK, Zhang Y, Guo J, Liang W, et al. Facilitating T cell infiltration in tumor microenvironment overcomes resistance to PD-L1 blockade. Cancer Cell. 2016;29(3):285–96.
194. Bassez A, Vos H, van Dyck L, Floris G, Arijs I, Desmedt C, et al. anti-PD1 treatment of patients with breast cancer. Nat Med. 2021; https://doi.org/10.1038/s41591-021-01323-8.
195. Adams S, Loi S, Toppmeyer D, Cescon DW, de Laurentiis M, Nanda R, et al. Pembrolizumab monotherapy for previously untreated, PD-L1-positive, metastatic triple-negative breast cancer: Cohort B of the phase II KEYNOTE-086 study. Ann Oncol. 2019;30(3):405–11.
196. Emens LA, Molinero L, Loi S, Rugo HS, Schneeweiss A, Diéras V, et al. Atezolizumab and nab -Paclitaxel in advanced triple-negative breast cancer: biomarker evaluation of the IMpassion130 study. J Natl Cancer Inst. 2021;
197. Foldi J, Silber A, Reisenbichler E, Singh K, Fischbach N, Persico J, et al. Neoadjuvant durvalumab plus weekly nab-paclitaxel and dose-dense doxorubicin/cyclophosphamide in triple-negative breast cancer. NPJ Breast Cancer. 2021;7(1):9.
198. Emens L, Loi S, Rugo H, Schneeweiss A, Dieras V, Iwata H, et al. IMpassion130: efficacy in immune biomarker subgroup from the global, randomized, double-blind, placebo-controlled, phase III study of atezolizumab + nab-paclitaxel in patients with treatment-naive, locally advanced or metastatic triple-negative breast cancer. Presented at the 2018 San Antonio breast cancer sympsium, San Antonio, TX, 4–7 December 2018.
199. Loi L, Winer E, Lipatov O, Im S, Goncalves A, Cortes J, et al. Relationship between tumor-infiltrating lymphocytes (TILs) and outcomes in the KEYNOTE-119 study of pembrolizumab vs chemotherapy for previously treated metastatic triple-negative breast

cancer (mTNBC). Philadelphia, PA: American Association for Cancer Research; 2020.
200. Loi S, Adams S, Schmid P, Cortes J, Cescon D, Winer E, et al. Relationship between tumor infiltrating lymphocyte levels and response to pembrolizumab in metastatic triple-negative breast cancer: Results from Keynote-086 trial. Presented at the European Society of Medical Oncology (ESMO) 2017 Congress, Madrid, Spain, 8–12 September 2017.
201. Emens LA, Esteva FJ, Beresford M, Saura C, de Laurentiis M, Kim SB, et al. Trastuzumab emtansine plus atezolizumab versus trastuzumab emtansine plus placebo in previously treated, HER2-positive advanced breast cancer (KATE2): a phase 2, multicentre, randomised, double-blind trial. Lancet Oncol. 2020;21(10):1283–95.
202. Loibl S, Untch M, Burchardi N, Huober J, Sinn BV, Blohmer JU, 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 study. Ann Oncol. 2019;30:1279–88.
203. Bianchini G, Huang C, Egle D, Bermejo B, Zamagni C, Thill M, et al. Tumour infiltrating lymphocytes (TILs), PD-L1 expression and their dynamics in the NeoTRIPaPDL1 trial. Ann Oncol. 2020;31(Suppl. 4):S1142–215.
204. Dieci MV, Guarneri V, Bisagni G, Tosi A, Musolino A, Spazzapan S, et al. 162MO Neoadjuvant chemotherapy and immunotherapy in Luminal B BC: results of the phase II GIADA trial. Ann Oncol. 2020 Sep;31:S304–5.
205. Khoury T, Peng X, Yan L, Wang D, Nagrale V. Tumor-infiltrating lymphocytes in breast cancer: evaluating interobserver variability, heterogeneity, and fidelity of scoring core biopsies. Am J Clin Pathol. 2018;150(5):441–50.
206. Tramm T, Di Caterino T, Jylling AB, Lelkaitis G, Laenkholm AV, Rago P, et al. Standardized assessment of tumor-infiltrating lymphocytes in breast cancer: an evaluation of inter-observer agreement between pathologists. Acta Oncol. 2018;57(1):90–4.
207. Swisher SK, Wu Y, Castaneda CA, Lyons GR, Yang F, Tapia C, et al. Interobserver agreement between pathologists assessing tumor-infiltrating lymphocytes (TILs) in breast cancer using methodology proposed by the international TILs working group. Ann Surg Oncol. 2016;23(7):2242–8.
208. Amgad M, Stovgaard ES, Balslev E, Thagaard J, Chen W, Dudgeon S, et al. Report on computational assessment of tumor Infiltrating lymphocytes from the international immuno-oncology biomarker working group. NPJ Breast Cancer. 2020;6:16.
209. von Minckwitz G, Schneeweiss A, Loibl S, Salat C, Denkert C, Rezai M, et al. Neoadjuvant carboplatin in patients with triple-negative and HER2-positive early breast cancer (GeparSixto; GBG 66): a randomised phase 2 trial. Lancet Oncol. 2014;15(7):747–56.
210. Dieci MV, Radosevic-Robin N, Fineberg S, van den Eynden G, Ternes N, Penault-Llorca F, et al. Update on tumor-infiltrating lymphocytes (TILs) in breast cancer, including recommendations to assess TILs in residual disease after neoadjuvant therapy and in carcinoma in situ: a report of the International Immuno-Oncology Biomarker Working Group on Breast Cancer. Semin Cancer Biol. 2018;52(Pt 2):16–25.
211. Buisseret L, Desmedt C, Garaud S, Fornili M, Wang X, Van den Eyden G, et al. Reliability of tumor-infiltrating lymphocyte and tertiary lymphoid structure assessment in human breast cancer. Mod Pathol. 2017;30(9):1204–12.
212. Cha YJ, Ahn SG, Bae SJ, Yoon CI, Seo J, Jung WH, et al. Comparison of tumor-infiltrating lymphocytes of breast cancer in core needle biopsies and resected specimens: a retrospective analysis. Breast Cancer Res Treat. 2018;171(2):295–302.
213. Althobiti M, Aleskandarany MA, Joseph C, Toss M, Mongan N, Diez-Rodriguez M, et al. Heterogeneity of tumour-infiltrating lymphocytes in breast cancer and its prognostic significance. Histopathology. 2018;73(6):887–96.
214. Campbell BB, Light N, Fabrizio D, Zatzman M, Fuligni F, de Borja R, et al. Comprehensive analysis of hypermutation in human cancer. Cell. 2017;171(5):1042–1056.e10.
215. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017;357(6349):409–13.
216. Cheng AS, Leung SCY, Gao D, Burugu S, Anurag M, Ellis MJ, et al. Mismatch repair protein loss in breast cancer: clinicopathological associations in a large British Columbia cohort. Breast Cancer Res Treat. 2020;179(1):3–10.
217. Salgado R, Bellizzi AM, Rimm D, Bartlett JMS, Nielsen T, Holger M, et al. How current assay approval policies are leading to unintended imprecision medicine. Lancet Oncol. 2020;21:1399–401.
218. Litchfield K, Reading JL, Puttick C, Thakkar K, Abbosh C, Bentham R, et al. Meta-analysis of tumor- and T cell-intrinsic mechanisms of sensitization to checkpoint inhibition. Cell. 2021;184(3):596–614.
219. Raskov H, Orhan A, Christensen JP, Gögenur I. Cytotoxic CD8+ T cells in cancer and cancer immunotherapy. Br J Cancer. 2021;124:359–67.
220. Schmidt M, Weyer-Elberich V, Hengstler JG, Heimes AS, Almstedt K, Gerhold-Ay A, et al. Prognostic impact of CD4-positive T cell subsets in early breast cancer: a study based on the FinHer trial patient population. Breast Cancer Res. 2018;20(1):15.
221. Liu S, Lachapelle J, Leung S, Gao D, Foulkes WD, Nielsen TO. CD8 +lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. Breast Cancer Res. 2012;14(2):R48.
222. García-Martínez E, Gil GL, Benito AC, González-Billalabeitia E, Conesa MAV, García TG, et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. Breast Cancer Res. 2014;16(1):488.
223. Oda N, Shimazu K, Naoi Y, Morimoto K, Shimomura A, Shimoda M, et al. Intratumoral regulatory T cells as an independent predictive factor for pathological complete response to neoadjuvant paclitaxel followed by 5-FU/epirubicin/cyclophosphamide in breast cancer patients. Breast Cancer Res Treat. 2012;136(1):107–16.
224. Lee JS, Ruppin E. Multiomics prediction of response rates to therapies to inhibit programmed cell death 1 and programmed cell death 1 ligand 1. JAMA Oncol. 2019;5(11):1614–8.
225. Jiang W, He Y, He W, Wu G, Zhou X, Sheng Q, et al. Exhausted CD8+T cells in the tumor immune microenvironment: new pathways to therapy. Front Immunol. 2021;11:622509.
226. Speiser DE, Ho P-C, Verdeil G. Regulatory circuits of T cell function in cancer. Nat Rev Immunol. 2016;16(10):599–611.
227. Ma X, Xiao L, Liu L, Ye L, Su P, Bi E, et al. CD36-mediated ferroptosis dampens intratumoral CD8+ T cell effector function and impairs their antitumor ability. Cell Metab. 2021;33(5):1001–1012.e5.
228. Gruosso T, Gigoux M, Manem VSK, Bertos N, Zuo D, Perlitch I, et al. Spatially distinct tumor immune microenvironments stratify triple-negative breast cancers. J Clin Investig. 2019;129(4):1785–800.
229. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature. 2017;541:321–30.
230. Nawaz S, Heindl A, Koelble K, Yuan Y. Beyond immune density: Critical role of spatial heterogeneity in estrogen receptor-negative breast cancer. Mod Pathol. 2015;28(6):766–77.
231. Heindl A, Sestak I, Naidoo K, Cuzick J, Dowsett M, Yuan Y. Relevance of spatial heterogeneity of immune infiltration for predicting risk of recurrence after endocrine therapy of ER+ breast cancer. J Natl Cancer Inst. 2018;110(2)

232. Berben L, Wildiers H, Marcelis L, Antoranz A, Bosisio F, Hatse S, et al. Computerised scoring protocol for identification and quantification of different immune cell populations in breast tumour regions by the use of QuPath software. Histopathology. 2020;77(1):79–91.
233. Berben L, Floris G, Kenis C, Dalmasso B, Smeets A, Vos H, et al. Age-related remodelling of the blood immunological portrait and the local tumor immune response in patients with luminal breast cancer. Clin Transl Immunol. 2020;9(10):e1184.
234. Sobottka B, Pestalozzi B, Fink D, Moch H, Varga Z. Similar lymphocytic infiltration pattern in primary breast cancer and their corresponding distant metastases. OncoImmunology. 2016;5(6):e1153208.
235. Keren L, Bosse M, Marquez D, Angoshtari R, Jain S, Varma S, et al. A structured tumor-immune microenvironment in triple negative breast cancer revealed by multiplexed ion beam imaging. Cell. 2018;174(6):1373–1387.e19.
236. Nederlof I, Horlings HM, Curtis C, Kok M. A high-dimensional window into the micro-environment of triple negative breast cancer. Cancers. 2021;13(2):316.
237. Nederlof I, de Bortoli D, Bareche Y, Nguyen B, de Maaker M, Hooijer GKJ, et al. Comprehensive evaluation of methods to assess overall and cell-specific immune infiltrates in breast cancer. Breast Cancer Res. 2019;21(1):151.
238. de Smet F, Antoranz Martinez A, Bosisio FM. Next-generation pathology by multiplexed immunohistochemistry. Trends Biochem Sci. 2021;46:80–2.
239. Lun XK, Bodenmiller B. Profiling cell signaling networks at single-cell resolution. Mol Cell Proteomics. 2020;19:744–56.
240. Jackson HW, Fischer JR, Zanotelli VRT, Ali HR, Mechera R, Soysal SD, et al. The single-cell pathology landscape of breast cancer. Nature. 2020;578(7796):615–20.
241. Egelston CA, Avalos C, Tu TY, Rosario A, Wang R, Solomon S, et al. Resident memory CD8+ T cells within cancer islands mediate survival in breast cancer patients. JCI Insight. 2019;4(19)
242. Byrne A, Savas P, Sant S, Li R, Virassamy B, Luen SJ, et al. Tissue-resident memory T cells in breast cancer control and immunotherapy responses. Nat Rev Clin Oncol. 2020;17:341–8.
243. Reddy SM, Reuben A, Barua S, Jiang H, Zhang S, Wang L, et al. Poor response to neoadjuvant chemotherapy correlates with mast cell infiltration in inflammatory breast cancer. Cancer Immunol Res. 2019;7(6):1025–35.
244. Carvajal-Hausdorf DE, Patsenker J, Stanton KP, Villarroel-Espindola F, Esch A, Montgomery RR, et al. Multiplexed (18-Plex) measurement of signaling targets and cytotoxic T cells in Trastuzumab-treated patients using imaging mass cytometry. Clin Cancer Res. 2019;25(10):3054–62.
245. Griguolo G, Serna G, Pascual T, Fasani R, Guardia X, Chic N, et al. Immune microenvironment characterisation and dynamics during anti-HER2-based neoadjuvant treatment in HER2-positive breast cancer. NPJ Precis Oncol. 2021;5(1):23.
246. de Angelis C, Nagi C, Hoyt CC, Liu L, Roman K, Wang C, et al. Evaluation of the predictive role of tumor immune infiltrate in patients with HER2-positive breast cancer treated with neoadjuvant anti-HER2 therapy without chemotherapy. Clin Cancer Res. 2020;26(3):738–45.
247. Allam M, Cai S, Coskun AF. Multiplex bioimaging of single-cell spatial profiles for precision cancer diagnostics and therapeutics. NPJ Precis Oncol. 2020;4(1):11.
248. Klauschen F, Müller KR, Binder A, Bockmayr M, Hägele M, Seegerer P, et al. Scoring of tumor-infiltrating lymphocytes: from visual estimation to machine learning. Semin Cancer Biol. 2018;52:151–7.
249. Saltz J, Gupta R, Hou L, Kurc T, Singh P, Nguyen V, et al. Spatial organization and molecular correlation of tumor-infiltrating lymphocytes using deep learning on pathology images. Cell Rep. 2018;23(1):181–93 e7.
250. Basavanhally AN, Ganesan S, Agner S, Monaco JP, Feldman MD, Tomaszewski JE, et al. Computerized image-based detection and grading of lymphocytic infiltration in HER2+ breast cancer histopathology. IEEE Trans Biomed Eng. 2010;57(3):642–53.
251. Acs B, Ahmed FS, Gupta S, Wong PF, Gartrell RD, Sarin Pradhan J, et al. An open source automated tumor infiltrating lymphocyte algorithm for prognosis in melanoma. Nat Commun. 2019;10(1)
252. Chou M, Illa-Bochaca I, Minxi B, Darvishian F, Johannet P, Moran U, et al. Optimization of an automated tumor-infiltrating lymphocyte algorithm for improved prognostication in primary melanoma. Mod Pathol. 2021;34(3):562–71.
253. Yuan Y, Failmezger H, Rueda OM, Ali HR, Gräf S, Chin S-F, et al. Quantitative image analysis of cellular heterogeneity in breast tumors complements genomic profiling. Sci Transl Med. 2012;4(157):157ra43-ra43.
254. Le H, Gupta R, Hou L, Abousamra S, Fassler D, Kurc T, et al. Utilizing automated breast cancer detection to identify spatial distributions of tumor infiltrating lymphocytes in invasive breast cancer. Am J Pathol. 2020;190(7):1491–504.
255. Swiderska-Chadaj Z, Pinckaers H, van Rijthoven M, Balkenhol M, Melnikova M, Oscar G, et al. Convolutional neural networks for lymphocyte detection in immunohistochemically stained whole-slide images MIDL 2018 conference submission2018.
256. Yoon HH, Shi Q, Heying EN, Muranyi A, Bredno J, Ough F, et al. Intertumoral heterogeneity of CD3+ and CD8+ T-cell densities in the microenvironment of DNA mismatch-repair–deficient colon cancers: implications for prognosis. Clin Cancer Res. 2019;25(1):125–33.
257. Corredor G, Wang X, Zhou Y, Lu C, Fu P, Syrigos K, et al. Spatial architecture and arrangement of tumor-infiltrating lymphocytes for predicting likelihood of recurrence in early-stage non–small cell lung cancer. Clin Cancer Res. 2019;25(5):1526–34.
258. Amgad M, Elfandy H, Hussein H, Atteya LA, Elsebaie MAT, Abo Elnasr LS, et al. Structured crowdsourcing enables convolutional segmentation of histology images. Bioinformatics. 2019;35(18):3461–7.
259. Amgad M, Sarkar A, Srinivas C, Redman R, Ratra S, Bechert C, et al. Joint region and nucleus segmentation for characterization of tumor infiltrating lymphocytes in breast cancer. SPIE. 2019;
260. Tizhoosh HR, Pantanowitz L. Artificial intelligence and digital pathology: challenges and opportunities. J Pathol Inform. 2018;9:38.
261. Cui M, Zhang DY. Artificial intelligence and computational pathology. Lab Investig. 2021;101(4):412–22.