Medicine Updates Faculty of medicine
October 2022,volume 11, issue 11

https://muj.journals.ekb.egdean@med.psu.edu.eg
vice_dean_postgraduate@med.psu.edu.eg
DOI: 10.21608/muj.2022.124010.1093

ISSN : 2682-2741

Submitted: 2 /3/2022
Accepted : 8/6/2022
Pages:16 - 29

" OVERVIEW OF TUMOR INFILTRATING LYMPHOCYTES SIGNIFICANCE IN BREAST CANCER "

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Abstract:

Tumor growth and progression is characterized by a constant interplay of tumor cells with the host immune cells in tumor microenviroment. In human epidermal growth factor receptor (HER2)+ve as well as triple negative breast cancer (TNBC), immune infiltrates can be found in up to 75% of tumors. Almost one third of these cases show a significantly dense infiltrate of immune cells. On the other hand, luminal subtypes (hormone receptor positive) have the least amount of tumor infiltrating lymphocytes. These tumor infiltrating lymphocytes *(TILS)* reflect the host's attempt to eliminate malignancies, and over the past years, they have been shown to possess a promising prognostic value in various malignancies including breast cancer, particularly in triple negative and HER2 +ve molecular subsets. Moreover, the assessment of TILs percentage has been effective in predicting outcome of treatment regimens in neo/adjuvant settings. In the meantime, with introduction of novel immune-related therapeutic options, TILs continue to show further predictive utility which will soon be implemented in clinical practice. This implies a robust rationale for the fore coming therapies.

In this review article we are going to highlight the basics and recent studies the field of interactions between immune response and breast cancer.

Key words: Breast cancer; Tumor infiltrating lymphocytes; HER2+; Trastuzumab; TNBC.

Introduction

Tumor growth and progression is characterized by a constant interplay of tumor cells with the host immune cells in tumor microenviroment. In breast cancer, particularly human epidermal growth factor receptor (HER2)+ve as well as triple negative breast cancer (TNBC), immune infiltrates can be found in up to 75% of tumors. Almost one third of these cases show a significantly dense infiltrate of immune cells. On the other hand, luminal subtypes (hormone receptor positive) have the least amount of tumor infiltrating lymphocytes. ***(Stanton SE et al, 2016)***

These tumor infiltrating lymphocytes (TILS) reflect the host's attempt to eliminate malignancies, and over the past years, they have been shown to possess a promising prognostic value in various malignancies including breast cancer, particularly in triple negative and HER2 +ve molecular subsets. Moreover, the assessment of TILs percentage has been effective in predicting outcome of

treatment regimens in neo/adjuvant settings.***(Ravelli A et al, 2017)***

Biology

Breast tumors are associated with considerable infiltration by different leukocyte populations. This includes lymphocytes, neutrophils, macrophages and others. These cells are responsible for both passive and aquired immune responses. Lymphocytes in particular have been studied more broadly, being were likely to detect and possibly destroy malignant clones. On the other hand, a pathway that allows malignant cells to escape immune response and to thrive was assumed to occur. This hypothesis may, in part, clarify the unresponsiveness associated with immune-infiltrates. The mechanism that makes malignant cells able to evade immune-recognition and elimination is called as *immunoediting,* a process firstly explained by ***Dunn et al. in 2004***.

The process starts with "*elimination phase"* where immunosurveillance-related lymphocytes are able to identify and 'kill' cancer cells, thus opposing tumor cell growth. These lymphocytes include *CD4+* type-1 T-helper (Th-1) cells, *cytotoxic CD8+* T cells and natural killer (NK) cells. Over time, tumor cells gradually favor the proliferation of malignant clones which are more adapted to escape the immune system recognition and destruction through several biological tactics. Through reducing the expression of surface cancer antigens, malignant cells become *'invisible'* to detection by immune system, therefore exceedingly decreasing the immunogenicity of the lesion "*equilibrium* phase". Also, through overexpression of immune-checkpoint molecules, cancer cells become more efficient in inhibiting the activity of surrounding lymphocytes, as well as augmenting the proliferation and survival of immune-inhibitory cells *(myeloid derived suppressor cells (MDSCs) and FOXP3 regulatory T-cells (Tregs)*. This results in the "*escape-phase"*. The immune check-points, that normally functions to prevent host self-reactivation, later become a detrimental effect, resulting in more immune suppression. Following an *equilibrium* stage between eliminated and escaping tumor cells, the malignant cells become surrounded by mainly nonfunctional lymphocytes and immune-suppressive cells, therefore establishing the *escape* phase (.Figure 1). ***(Dunn et al, 2004 & Postow MA et al, 2012)***

The components of leukocytes infiltrating breast tumors was thoroughly analyzed by **Gu-*Trantien et al. (2013)***. The main component was T-lymphocytes constituting about three quarters of the population, B-lymphocytes were less than

fifth, monocytes constituted less than 10% of cells, and NK T-cells made up less than 5% of all leukocytes. ***(Gu-Trantien C et al, 2013)***

TILs may be classified according to tumor site localization into lymphocytes that infiltrate the tumor stroma *(stromal TILs)* and those infiltrating the tumor cell niches or in direct contact with tumor cells, called *intra-tumoral TILs* ***(Hornychova H et al, 2008)***

Variations were also found according to different breast cancer histologies. Negative hormone receptors subtype was shown to have the highest TILs levels. Also, triple negative subtype, high tumor grade as well as high expression of Ki-67proliferation antigen were found to have similar finding of high infiltration level. ***(Seo AN et al, 2013)***.

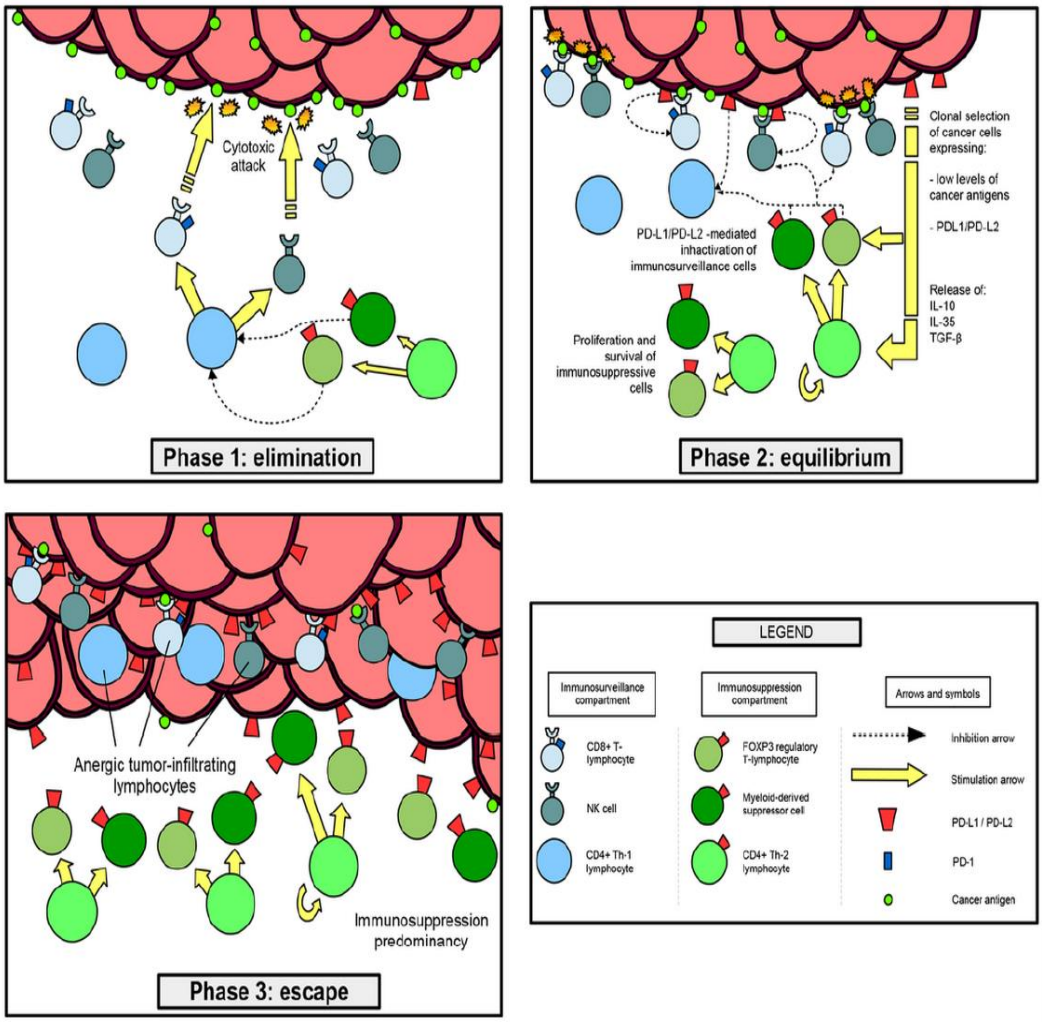

Figure 1. Illustration of the immune-editing theory.

In the majority of clinical trials, stromal TILs have shown to provide more robust and representable markers than intratumoral TILs. As previously mentioned, the

majority of TILs are *T- lymphocytes* whereas B Lymphocytes are less numerous. The role of B lymphocytes (CD20+ve) in tumor immune response is still unclear. ***(Schmidt M et al, 2012).*** Other several interactions have been shown to implement the immune tumor response. CD3+ve TILs showed an activated phenotype as well as increased expression of CD69 and human leucocyte antigen-DR (HLA DR) which are considered as activation markers, in addition to several chemokine receptors including CXC receptor 3, CCR4 and CCR5. Moreover, there is a linkage between the expression of chemokine CXCL10 / CXCR3 receptor and TILs including CD8 /CD4/ FOXP3 ***(Mulligan et al. 2013).***

Factor affecting TILs recruitment

Several factors are involved in lymphocyte recruitment in tumor microenviroment. In breast cancer, the presence of high endothelial venules*(HEV)* may contribute to lymphocytic attraction by interacting with blood vessel wall. There was a relatively improved survival outcome in patients having high density of HEV which is correlated to *lymphotoxin-β* produced by mature dendritic cells. It was also noticed that the density of HEV is higher in ductal carcinoma insitu compared to invasive ductal carcinoma. ***(Martinet L et al, 2013)***

Indoleamine-2,3-dioxygenase *(IDO)* is an important enzyme affecting lymphocytic infiltration in tumors. It catalyzes the change of tryptophan to kynurenine. Kynurenine has cytotoxic antitumoral effect, while tryptophan depletion is associated with inhibition of both tumor cells and lymphocytes. This catalytic activity of *IDO* may result in inhibition or stimulation of tumor growth as well as antitumor immunological response. ***(Jacquemier J et al, 2012)***

Many other factors affecting both TILs count and recruitment have been explored. As mentioned earlier, TNBC is associated with high TIL count. The presence of TILs has been found to indirectly correlate with expression of *human leukocyte antigen-G*, which might be responsible for tumor evasion. There is also an association between TIL count and overexpression of *stem cell makers or epithelial mesenchymal transition markers* in malignant cells, however their significance is not yet clear. ***(Seo AN et al, 2013)***

TILs in clinical practice

Immunohistochemistry is the most commonly used method to detect TILs. This approach uses paraffin-embedded tumor specimens. It involves using monoclonal antibodies to detect the different lymphocyte subsets. Then, lymphocytes are visualized using secondary antibodies and signal amplification

systems which are then counted using a light microscope.

The most important advantage of this technique is the simplicity without affecting its accuracy. It can be carried out in virtually any pathology laboratory with routine immune-histochemical analysis. However, the main problem of TILs counting is that it requires pathologists with high expertise compared to other biomarkers (e.g. *hormonal receptors or HER2*) used in breast cancer. As a result, TIL assessment has not gained global use in routine practice despite the solid data that support its use, and TILs analysis remains bounded to investigational studies. Nonetheless, the immune-histochemical assessment of TILs is characteristic, compared to other techniques as it allows for classification of subgroups of TILs in separate niches of the malignant tissue *(stromal-versus-intratumoral). (**Melichar B et al, 2014).*** A standardized method for TILs assessment is established by the International TILS Working Group. (Fig.2)

On H&E-stained sections, stromal TILs tend to be more numerous and productive than intra-tumoral counterpart. The intratumoral TILs are usually less frequent and found in much fewer percentages, they tend to be less homogenous and harder to detect on routine H&E slides. Another advantage of stromal TILs over intratuomral counterpart is that the growth pattern and density of cancer cell niches do not affect the TILs count as stromal TILs are counted only in the areas in between the malignant cell nests.

In clinical setting, the international *TILs Working Group standard guide* has classified stromal TILs into: absent (0-10%)- intermediate (11-40%) and extensive (41-90%). This simple scheme can be easily implemented in clinical setting. (***Salgado R et al,2015)***

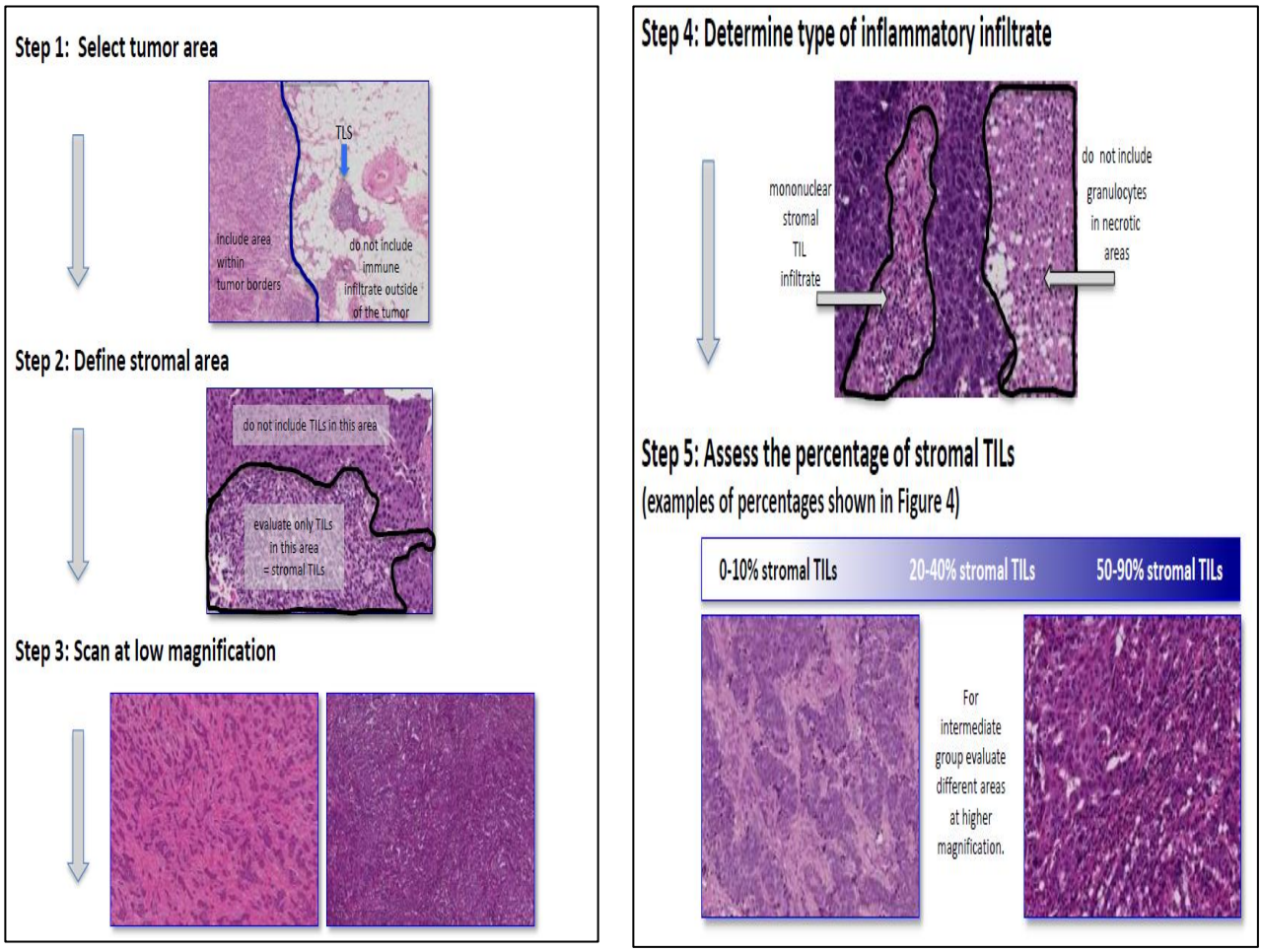

Fig.2 TILs evaluation guideline recommended by the International TILS Working-Group.

Lymphocyte-predominant breast cancer (LPBC) is another group which includes tumors that have more than 50% lymphocyte infiltration and it carries the best prognosis. Several studies reported different values for LPBC with various percentages, however in one study an LPBC with 60% TILs was found in 28% of triple negative and 20% of HER2-enriched breast cancer cases. Both subtypes were associated with better prognosis compared to other cases with less TIL percentages ***(Pruneri G., et al 2015)***.

Clinical outcome is also affected by the phenotype of the infiltrate. CD8 +ve cytotoxic T cells *(CTL)* play an important role in tumor elimination, type 1 T cells *(Th1)* are associated with favorable outcome by facilitating antigen presentation. This occurs by activating antigen presenting cells *(APC)* and secretion of several cytokines. On the other hand, type 2 CD4 +ve T helper cells *(Th2)*, including Forkhead box P3 *(FOXP3)* CD4 +ve T cells, inhibit CTL function, and may trigger an anti inflammatory response which may increase tumor growth. ***(Salgado R et al, 2015)***

Role of TILs in Predicting Response to Neoadjuvant Systemic Therapy

With the current increasing adoption of neoadjuvant therapy, it allows for a prompt histological analysis of the therapeutic efficacy of the used treatment protocol. The predictive role of several biomarkers, including TILs can now be evaluated. It also gives an opportunity to assess biomarker changes in pre and post treatment settings. In breast cancer, neoadjuvant chemotherapy is associated with changes of laboratory parameters that reflect an immune response; this includes rising numbers of circulating T-lymphocytes. ***(Melichar B et al, 2008)***

In a small cohort study, the numbers of stromal CD3+ TILs as well as dendritic cells *(CD83+)* in pretreatment tissue sample were notably higher in cases who later on achieved pathological complete response *(pCR)* ***(Hornychova H et al, 2008).***

The predictive role of TILs on pathological response was subsequently assessed and confirmed on a larger group of patients. In GeparDuo and GeparTrio trials, analysis was done in more than 1000 patients. The percentage of tumor epithelial nests containing stromal lymphocytes was an independent predictor of pCR in the multivariate analysis. The pCR rate was 40% in patients with tumors extensive infiltrate (LPBC), compared to less than 8% in cases with tumors showing absent lymphocytic infiltrate ***(Denkert C et al, 2010)***

According to breast cancer subtype, the level of TILs varies widely. Generally, TILs has been listed to be associated with ductal histologies, high grade, negative hormone receptors, HER2 positive and those with high proliferation antigen Ki67 expression ***(Loi S et al, 2013).*** High lymphocyte infiltration is significantly observed in cases of TNBC, which may signify the high pCR rate observed in this subtype ***(Rapoport et al., 2020).*** Moreover, the relation between TILs and pathological response to neoadjuvant chemotherapy in TNBC and other histological variants has been studied in several trials. There was a statistically significant correlation between TIL percentage and pathological response. It was more evident in TNBC, but not in other histological subtypes. In cases with hormone receptor negative tumors, the high expression of TILs was reported to be correlated with pCR in patients treated with neoadjuvant anthracycline based chemotherapy. Also, in cases with both HER2 enriched and TNBC, there was a higher number of TILs found in patients who achieved pCR ***(West NR et al, 2011).***

FOXP3+TILs was found to be an independent predictor of pCR in multivariate analysis. In patients achieving pCR ***Seo AN et al.*** noticed a larger number of

CD8+, CD4+ and FOXP3+ve TILs ***(2013*)**. CD8+ TILs were also independent predictors of pCR.

Furthermore; in the PREDICT study were more than three hundred breast cancer, the predictive significance of TILs was confirmed ***(Issa-Nummer Y et al, 2013)***. LPBC and stromal TILs were both independent predictive factors of pCR in multivariate analysis, while in univariate analysis, intra-tumoral lymphocytes were associated with pCR.

The reasonable explanation of predictive significance of TILs and pathological response to chemotherapy is yet to be established. Several hypotheses have been generated in this field. Chemotherapy causes decrease in tumor burden which facilitates an antitumor immune response enabling a more potent immune response. Also, some chemotherapeutic drugs like anthracyclines have a direct antitumor effect through inducing an immunogenic tumor cell autolysis. Chemotherapy can amplify immune response by depleting inhibitory T cells and myeloid derived suppressor cells that normally function by inhibiting antitumor immune response. Furthermore, several somatic mutations can be induced by chemotherapy resulting in novel peptide antigens that appear hostile to the immune system, therefore masking immune recognition. ***(Tung NM et al, 2015)*** Till now, the role of tumor lymphocytes is limited to prognosis and predicting response of a given treatment but is it does not help in the selection of chemotherapy.

TIL as Prognostic Biomarker in Breast Cancer

Several studies have shown a relationship between number of TILs and prognosis in breast cancer. In the Breast International Group 02-08 trial, analysis of more than two thousand breast cancer cases demonstrated that higher percentages of TILs (both intra-tumoral and stromal) were associated with better disease free survival (DFS) as well as overall survival (OS) particularly in cases with TNBC subtype ***(Loi S et al, 2013)***

In another study were 256 patients with TNBC, those with LPBC achieved much higher 5 year survival rate reaching 92% compared to 71% in patients with less intense infiltration. Both values were remarkably high. In another study of more than one thousand breast cancer cases, higher CD8+ve TILs were correlated with better outcome ***(Mahmoud SMA et al, 2011)***

In untreated TNBC, improved survival was correlated with high TILs value, as

well as better disease free survival, and lower incidence of metastasis. While in HER2 enriched breast cancer, the presence of TILs is correlated with risk reduction of breast cancer mortality. ***(Cimino-Mathews A et al, 2015)***

West et al. (2013) reported that high levels of FOXP3+ve TILs were associated with better prognosis in hormone receptor negative tumors. *FOXP3+ve TILs* was also found to be correlated with CD8+ve TILs ***(West NR et al, 2013)***

It was found that the presence of programmed death *(PD)-1+ve* TILs had a negative impact on survival outcome in breast cancer. This was noticed in patients with basal-like subtype and luminal-B tumors *(independent of HER-2 expression)*. In multivariate analysis PD-1+ TILs were an independent prognostic factor. ***(Muenst S et al, 2013)***

TIL and Immunotherapy in Breast Cancer

Till now, the role of immunotherapy has not yet proven its potency in most breast cancer subtypes apart from TNBC. Systemic therapy includes mainly cytotoxic chemotherapy, hormonal and targeting agents. Some of these drugs may play a role in manipulating the immune response. Trastuzumab, for example, its activity may be partially explained by the inducing antibody dependent cell mediated cytotoxicity *(ADCC)* ***(Henriques B et al, 2021)***. It was also shown that specific polymorphisms of immunoglobulin receptors are associated with increased induction of ADCC; this in turn was associated with significantly longer progression free survival. ***(Denkert C et al, 2018)***

Bevacizumab, an antiangiogenic drug, is a monoclonal antibody against vascular endothelial growth factor *(VEGF).* It may also increase the antitumor activity of chemotherapy by suppressing the immune response ***(Bear HD et al, 2012).*** In clinical practice, the immunological role of bevacizumab in augmenting antitumor response in breast cancer has not been yet proven. Other drugs inducing an effective immune response are recently incorporated into treatment protocols. Examples include: nivolumab which is a monoclonal antibody against PD-1 and ipilumumab an anti-cytotoxic T-lymphocyte antigen *(CTLA)-4*. Furthermore, better understanding of pathogenesis may be fruitful in expanding the roles and indications for drugs already in use in breast cancer. Denosumab is a monoclonal antibody against RANK ligand and is used in bone metastasis. The *RANK*-ligand is produced by *FOXP3+ve TILs* and its role in the progression of disease may define another mechanism of activity against tumors. ***(Topalian SL et al, 2012).***

Due to few immunotherapeutic options currently available in breast cancer

treatment, the position of TILs as biomarkers of immune response to drugs focused on the immune-system presently stays unclear. However, for patients treated with neoadjuvant chemotherapy the predictive role of TILs is seemingly evident based on the published data. It is also clear that the immune-system plays a significant role in the anti-tumor response of regular cytotoxic agents.

Conclusions

Tumor infiltrating lymphocytes poses a significant prognostic value and offers a reliable tool in assessment of disease free survival in triple negative and HER2 enriched breast cancer with less prognostic impact on luminal subtypes. More studies are needed for better incorporation of these data in clinical practice.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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