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Review

REVIEW

Relevance of Tumor-Infiltrating Immune Cell Composition and Functionality for Disease Outcome in Breast Cancer

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Abstract

Background: Not all breast cancer patients benefit from neoadjuvant or adjuvant therapy, resulting in considerable undertreatment or overtreatment. New insights into the role of tumor-infiltrating immune cells suggest that their composition, as well as their functionality, might serve as a biomarker to enable optimal patient selection for current systemic therapies and upcoming treatment options such as immunotherapy.
Methods: We performed several complementary unbiased in silico analyses on gene expression profiles of 7270 unrelated tumor samples of nonmetastatic breast cancer patients with known clinical follow-up. CIBERSORT was used to estimate the fraction of 22 immune cell types to study their relations with pathological complete response (pCR), disease-free survival (DFS), and overall survival (OS). In addition, we used four previously reported immune gene signatures and a CD8+ T-cell exhaustion signature to assess their relationships with breast cancer outcome. Multivariable binary logistic regression and multivariable Cox regression were used to assess the association of immune cell–type fractions and immune signatures with pCR and DFS/OS, respectively.
Results: Increased fraction of regulatory T-cells in human epidermal growth factor receptor 2 (HER2)–positive tumors was associated with a lower pCR rate (odds ratio [OR] = 0.15, 95% confidence interval [CI] = 0.03 to 0.69), as well as shorter DFS (hazard ratio [HR] = 3.13, 95% CI = 1.23 to 7.98) and OS (HR = 7.69, 95% CI = 3.43 to 17.23). A higher fraction of M0 macrophages in estrogen receptor (ER)–positive tumors was associated with worse DFS (HR = 1.66, 95% CI = 1.18 to 2.33) and, in ER-positive/HER2-negative tumors, with worse OS (HR = 1.71, 95% CI = 1.12 to 2.61). Increased fractions of $\gamma\delta$ T-cells in all breast cancer patients related to a higher pCR rate (OR = 1.55, 95% CI = 1.01 to 2.38), prolonged DFS (HR = 0.68, 95% CI = 0.48 to 0.98), and, in HER2-positive tumors, with prolonged OS (HR = 0.27, 95% CI = 0.10 to 0.73). A higher fraction of activated mast cells was associated with worse DFS (HR = 5.85, 95% CI = 2.20 to 15.54) and OS (HR = 5.33, 95% CI = 2.04 to 13.91) in HER2-positive tumors. The composition of relevant immune cell types frequently differed per breast cancer subtype. Furthermore, a high CD8+ T-cell exhaustion signature score was associated with shortened DFS in patients with ER-positive tumors regardless of HER2 status (HR = 1.80, 95% CI = 1.07 to 3.04).
Conclusions: The main hypothesis generated in our unbiased in silico approach is that a multitude of immune cells are related to treatment response and outcome in breast cancer.

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Breast cancer outcome has clearly improved in recent decades. Advances in neoadjuvant and adjuvant treatment have contributed in large part to this progress. However, not all patients benefit from standard treatment regimens (1,2), resulting in undertreatment or overtreatment in many women. Predicting treatment response is particularly challenging for upcoming treatment options such as immunotherapy (3,4), especially in view of the potentially severe side effects of immunotherapeutic drugs. Consequently, optimal patient selection for systemic therapy is crucial.

Breast cancer has long been thought of as a nonimmunogenic malignancy, but a growing body of evidence suggests that this might not always be the case. The most widely studied immune cells in this context are tumor-infiltrating lymphocytes (TILs). Presence of TILs has been shown to be potentially predictive and prognostic in specific breast cancer subtypes. Specifically in patients with human epidermal growth factor receptor 2 (HER2)–positive and triple-negative breast cancer (TNBC), large adjuvant studies have shown that higher levels of TILs in primary biopsies are associated with improved overall survival (OS) and fewer recurrences, regardless of therapy (5–7). In patients with TNBC and HER2-positive tumors, increased levels of TILs are also associated with a higher pathological complete response (pCR) rate following neoadjuvant therapy (8–10). Moreover, patients with HER2-positive breast cancer and higher levels of TILs benefit more from adjuvant trastuzumab treatment (6).

Besides lymphocytes, tumors commonly contain tumor-associated macrophages (TAMs). In breast cancer patients, these TAMs have been associated with a shorter disease-free survival (DFS) and OS (11–13). TILs and TAMs are thus potential biomarkers. In addition, several broader immune gene signatures have been developed and related to breast cancer outcome (14–17).

However, the number of TILs does not always predict response to treatment, indicating that additional factors play a role. One possibility is that the functionality of various tumor-infiltrating immune cells should also be taken into account. For example, a CD8+ T-cell exhaustion signature, developed in purified circulating CD8+ T-cells, has recently been related to favorable prognosis of patients with autoimmune and inflammatory disease (18). It is still unknown whether CD8+ T-cell exhaustion might also be relevant in tumors as a possible explanation for tumor immune evasion.

These new insights into the role of tumor-infiltrating immune cells suggest that their composition as well as their functionality might be relevant for breast cancer management. In the present study, we therefore performed several complementary unbiased in silico analyses in an extensive data set comprising gene expression profiles of 7270 unrelated tumor samples of nonmetastatic breast cancer patients with known clinical follow-up and 172 normal breast samples from women without breast disease. In this hypothesis-generating study, we used CIBERSORT (19) to estimate the fractions of 22 immune cell types, which enabled us to study their independent associations with pCR, DFS, and OS in breast cancer in general and its subtypes in a large number of patients. In addition, we assessed the relationships with breast cancer outcome of four previously identified immune gene signatures (14–17) and a CD8+ T-cell exhaustion signature (18).

Methods

Detailed methods information is provided in the Supplementary Methods (available online).

Data Acquisition

Publicly available raw microarray expression data from newly diagnosed primary tumors of nonmetastasized breast cancer patients (prior to any treatment) and normal breast tissue were collected from the Gene Expression Omnibus (GEO), as well as relevant clinicopathological data and information on treatment regimen, pCR, and survival, whenever available (20). Analysis was confined to samples hybridized to the HG-U133A (GEO accession number GPL96) or Affymetrix HG-U133 Plus 2.0 (GEO accession number GPL570) platforms. Preprocessing and aggregation of raw data was performed according to the robust multi-array average algorithm. Quality control of the resulting expression data was executed as previously described (21–23).

Clinicopathological Data Collection

Information was collected on age, tumor histiotype, grade, tumor size, TNM stage, lymph node involvement, ER, progesterone receptor and HER2 status, treatment regimen, pCR, DFS, and OS. Data on ER, progesterone receptor status, and HER2 status was collected and scored according to immunohistochemistry staining guidelines of the American Society of Clinical Oncology and College of American Pathologists (24,25). Whenever immunohistochemistry data for receptor status were not reported, we determined receptor status by means of inference (see details in the Supplementary Methods, available online). For the treatment regimen, we labeled all samples with missing information about treatment as a separate category ("unknown"). DFS was defined as the interval between date of diagnosis until date of development of distant metastasis. OS was defined as the interval between date of diagnosis until date of death from any cause. The number of samples we used to assess the independent predictive and prognostic value of immune cell–type fractions, immune signatures, and CD8+ T-cell exhaustion signatures in breast cancer in general and in subtypes are provided in Supplementary Table 1 (available online).

Breast Cancer Subtypes

We performed analyses in several breast cancer subtypes based on receptor status and in the intrinsic molecular subtypes as defined by Sorlie et al., Parker et al., and Hu et al. (26–28). In addition, Lehmann et al. described seven TNBC subgroups that were identified by means of cluster analysis of gene expression profiles: basal-like 1, basal-like 2, unstable, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor (29). We applied the Lehmann classification to the collected TNBC tumors in order to compare estimated immune cell–type fractions within TNBC subgroups.

Estimated Immune Cell Type Fractions

CIBERSORT is a method for characterizing cell composition of complex tissues from their gene expression profiles that has been shown to have strong agreement with ground truth assessments in bulk tumors (19,30). We used the leukocyte gene signature matrix, termed LM22, which contains 547 genes that distinguish 22 human hematopoietic cell phenotypes, including seven T-cell types, naïve and memory B cells, plasma cells, natural killer (NK) cells, and myeloid subsets. We used CIBERSORT in combination with the LM22 signature matrix to estimate the fractions of 22 immune cell types in our collected breast cancer

and normal breast samples. For each sample, the sum of all estimate immune cell–type fractions equals 1.

Immune Gene Signatures

We investigated the relationships between immune cell–type fractions and four published immune signatures. Desmedt et al. identified an immune response gene signature associated with prognosis in HER2-positive and ER-negative/HER2-negative breast cancer subtypes (14). Teschendorff et al. determined that downregulation of a seven-gene immune signature was related to a higher risk of distant metastases in patients with ER-negative breast cancer (15). Perez et al. identified a set of immune function genes that may provide a means of predicting benefit from adjuvant trastuzumab treatment (16). Gu-Trantien et al. defined an eight-gene CD4+ follicular helper T-cell signature (Tfh signature) that predicted pathological tumor response following neoadjuvant therapy or survival (17). To compute the immune signature scores—often derived from gene signatures developed on other microarray platforms—for various data sets (distinct patient cohorts and laboratories), we used the weighted average method previously described (31). We only evaluated tumors that were hybridized to the Affymetrix HG-U133 Plus 2 platform. This ensured that we could use almost all genes that were part of individual immune signatures to calculate the scores.

Statistical Analysis

Distributions of the estimated immune cell–type fraction in normal breast tissue samples and breast cancer samples were compared by Mann-Whitney U test. All areas under the curves (AUCs) were rescaled within a range from -0.5 to 0.5. A negative AUC represented a relatively lower fraction of immune cell type in breast cancer compared with normal breast tissue, whereas a positive AUC represented a relatively higher fraction of an immune cell type in breast cancer.

The predictive value of estimated immune cell–type fractions in the neoadjuvant setting was assessed by multivariable binary logistic regression using pCR as outcome variable and age, T-stage (because of a low number of reported tumor size), grade, lymph node involvement, ER status, HER2 status, and treatment regimen as covariates. The prognostic value of estimated immune cell–type fractions in neoadjuvant and adjuvant settings was assessed by multivariable Cox regression analysis with time to distant metastasis and time to death as outcome variables and age, tumor size, grade, lymph node involvement, ER status, HER2 status, and treatment regimen as covariates. We used the listwise deletion method for handling of missing data. With this method, an entire sample is excluded from analysis if any single value is missing for the variables used in the multivariable Cox regression and multivariable binary logistic regression. Analyses were performed within a multivariable permutation testing framework for controlling the proportion of false discovery (32). For each breast cancer subset analysis, we used the multivariable permutation testing framework with 100 permutations and a false discovery rate (FDR) of 25%. An FDR of 25% indicates that the result is likely to be valid three out of four times. All results were considered statistically significant when P values were less than .05. All statistical tests were two-sided.

Results

Data Set Containing 7270 Breast Cancer Samples and 172 Normal Breast Tissue Samples

A summary of available baseline patient and primary tumor characteristics is presented in Table 1. We also assembled a reference group of 172 normal breast tissue samples obtained during reduction mammoplasty. Samples are classified according to their inferred ER and HER2 status, intrinsic molecular subtype (26–28), or TNBC subgroup as defined by Lehmann et al. (Figure 1) (29).

Composition of Tumor-Infiltrating Immune Cells

Figure 2 shows the immune cell composition in normal breast tissue versus breast cancer tissue (subtypes). Detailed results are provided in Supplementary Tables 2–4 (available online). Compared with normal breast tissue, breast cancer tissue generally contained a higher fraction for macrophages M0 (AUC = .34) and M1 (AUC = .22), T-cells follicular helper (AUC = .21), and regulatory T-cells (AUC = .28), whereas the plasma cell fraction was lower (AUC = −.25) (Figure 2, left box). This pattern was similar for receptor-based breast cancer subtypes compared with normal breast tissue. Within the intrinsic molecular subtypes, especially

Table 1. Baseline patient and primary tumor characteristics*

Variable	No. of samples	%	Valid %
Age at diagnosis, y			
≤50	1854	25.5	43.5
>50	2408	33.1	56.5
Missing	3008	41.4	
Tumor grade			
1	406	5.6	13.4
2	1260	17.3	41.5
3	1370	18.8	45.1
Missing	4234	58.2	
T-stage			
T0	8	0.1	0.3
T1	445	6.1	16.5
T2	1466	20.2	54.2
T3	467	6.4	17.3
T4	306	4.2	11.7
Missing	4578	63.0	
Lymph node involvement			
True	2134	29.4	45.3
False	4715	35.5	54.7
Missing	2555	35.1	
Stage			
I	193	2.7	10.7
II	1038	14.3	57.6
III	537	7.4	29.8
IV	35	0.5	1.9
Missing	5467	75.2	
ER status			
Positive	1294	17.8	73.1
Negative	476	6.5	26.9
Missing	5500	75.7	
HER2 status			
Positive	388	5.3	46.4
Negative	448	6.2	53.6
Missing	6434	88.5	

*ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2.

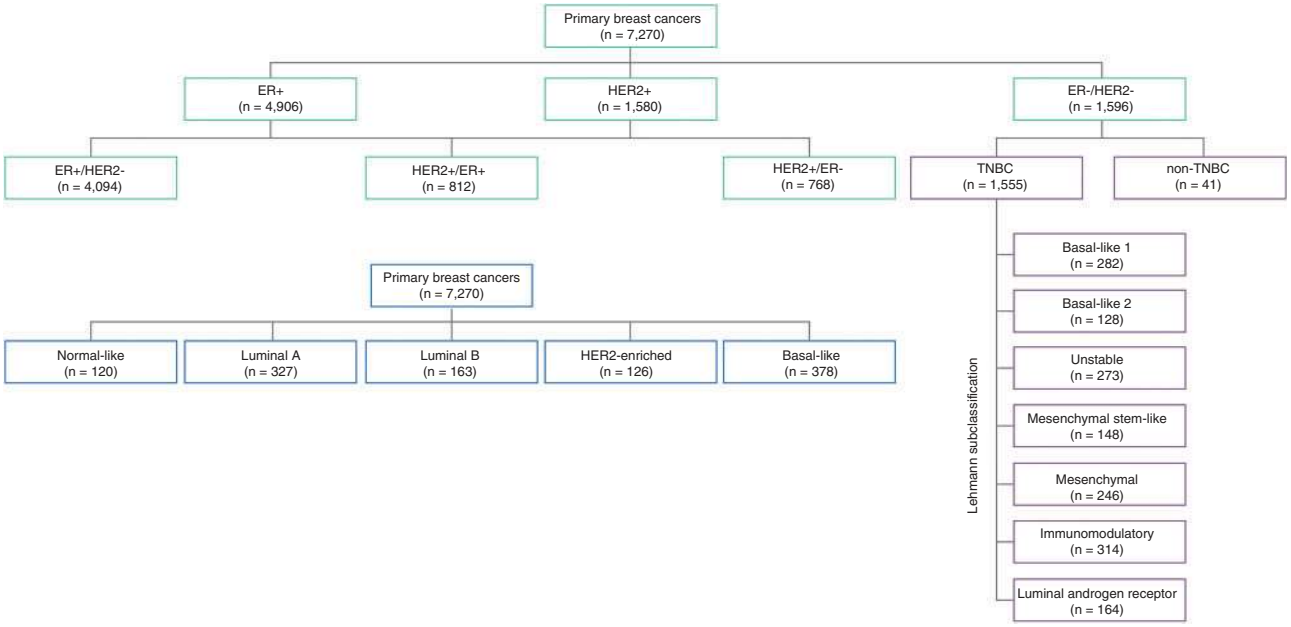

Figure 1. Overview of breast cancer subtypes based on inferred receptor status, intrinsic molecular subtype, and triple-negative breast cancer subgroup classification. TNBC subgroups are classified as defined by Lehmann et al. (29). The estrogen receptor (ER)–positive (n = 4906) and human epidermal growth factor receptor 2 (HER2)–positive (n = 1580) subtypes contain double cases, being the ER-positive/HER2-positive tumors (n = 812). ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; TNBC = triple-negative breast cancer.

HER2 and the basal subtype showed an increased fraction of macrophages M1 (AUC = .26 and AUC = .24, respectively). A relatively lower plasma cell fraction (AUC = −.11 was seen in HER2 subtype compared with the other intrinsic molecular subtypes. Within the Lehmann TNBC subgroups, the $\gamma\delta$ T-cell fraction was higher (AUC = .11) in the immunomodulatory subgroup compared with normal breast tissue (and relative to the other TNBC subgroups), whereas it was lower in the mesenchymal subgroup (AUC = -.17). The CD8+ T-cell fraction was highest in the immunomodulatory (AUC = .17) and luminal androgen receptor (AUC = .16) subgroups.

Immune Cell–Type Fractions as Independent Predictive or Prognostic Factors

Figure 3 shows the statistical significance of all immune cell–type fractions as independent predictive or prognostic factors for breast cancer subtypes. In the bubble heat map, a blue bubble indicates that a higher fraction is associated with lower pCR rate, shorter DFS, or shorter OS; a yellow bubble indicates that a higher fraction is associated with higher pCR rate, prolonged DFS, or prolonged OS. The size of a bubble indicates the statistical significance level. Detailed results are provided in Supplementary Tables 5–11 (available online). For regulatory T-cells, in the HER2-positive subtype (Supplementary Table 7, available online), a higher fraction was associated with a lower pCR rate (odds ratio [OR] = 0.15, 95% confidence interval [CI] = 0.03 to 0.69), worse DFS (hazard ratio [HR] = 3.13, 95% CI = 1.23 to 7.98), and worse OS (HR = 7.69, 95% CI = 3.43 to 17.23). A higher fraction of $\gamma\delta$ T-cells was associated with a higher pCR rate (OR = 1.55, 95% CI = 1.01 to 2.38) and prolonged DFS (HR = 0.68, 95% CI = 0.48 to 0.98), independent of receptor status (Supplementary Table 5, available online) and OS in the HER2-positive subtype (HR = 0.27, 95% CI = 0.10 to 0.73) (Supplementary Table 7, available online). For macrophages M1, a higher fraction was associated with a higher pCR rate (particularly in ER-positive disease; OR = 3.65, 95% CI = 1.51 to 8.82) (Supplementary Table 6, available online), as well as prolonged DFS (irrespective of subtype; HR = 0.53, 95% CI = 0.35 to 0.80) (Supplementary Table 5, available online). In the HER2-positive/ER-positive subtype (Supplementary Table 8, available online), a higher macrophage M1 fraction was most prominently associated with improved OS (HR = 0.22, 95% CI = 0.05 to 0.93). However, the opposite association was observed for a higher macrophage M0 fraction, particularly in ER-positive disease (irrespective of HER2 status) (Supplementary Table 6, available online) with DFS (HR = 1.66, 95% CI = 1.18 to 2.33), and for ER-positive/HER2-negative tumors with OS (HR = 1.71, 95% CI = 1.12 to 2.61) (Supplementary Table 9, available online). A higher activated mast cell fraction was associated with worse DFS and OS, most clearly in HER2-positive disease (HR = 5.85, 95% CI = 2.20 to 15.54, and HR = 5.33, 95% CI = 2.04 to 13.91, respectively) (Supplementary Table 7, available online). Also in HER2-positive disease (Supplementary Table 7, available online), a higher activated NK cell fraction was associated with prolonged DFS (HR = 0.39, 95% CI = 0.16 to 0.97), whereas a higher resting NK cell fraction indicated the opposite (HR = 3.73, 95% CI = 1.30 to 10.68). In addition, in the TNBC subtype (Supplementary Table 11, available online), a higher fraction of resting NK cells was also associated with worse DFS (HR = 18.91, 95% CI = 3.05 to 117.14) and OS (HR = 19.65, 95% CI = 1.66 to 232.56). For plasma cells, a higher fraction was associated with improved DFS (HR = 0.59, 95% CI = 0.40 to 0.88) regardless of receptor status (Supplementary Table 5, available online).

Immune Signatures as Independent Predictive or Prognostic Factors

Figure 4 shows the statistical significance of immune signatures as independent predictive or prognostic factors. Detailed results are provided in Supplementary Tables 12–14 (available online). A higher Tfh signature score was more statistically significantly associated in breast cancer (irrespective of receptor

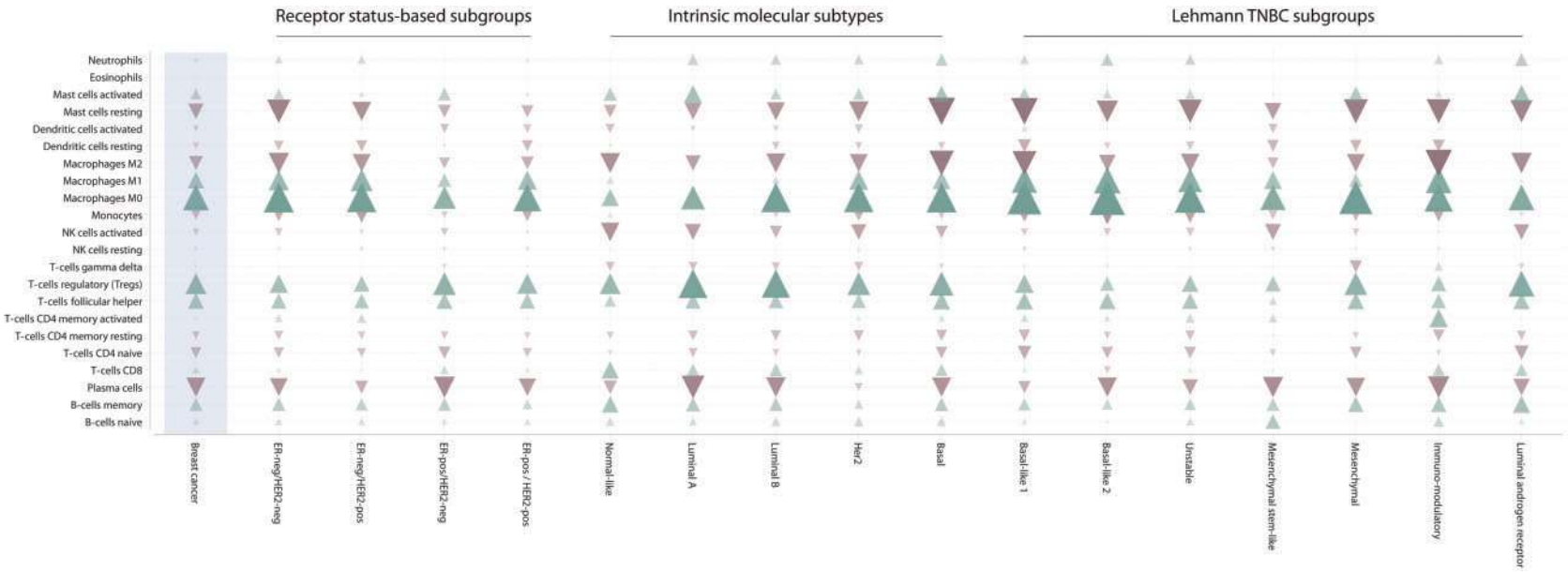

Figure 2. Distribution of immune cell–type fractions in breast cancer subtypes compared with healthy breast tissue. Fractions of each immune cell type were compared by means of two-sided Mann-Whitney U test for breast cancer subtypes based on inferred receptor status, intrinsic molecular subtypes, and triple-negative breast cancer subgroups as defined by Lehmann et al. (23). A **green triangle** indicates a higher immune cell–type fraction in breast cancer as compared with normal breast tissue. A **purple triangle** indicates a lower fraction in breast cancer as compared with normal breast tissue. The **size of the triangle** represents the area under the curve of the effect size of the shift in the distribution of immune cell–type fractions. ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; TNBC = triple-negative breast cancer.

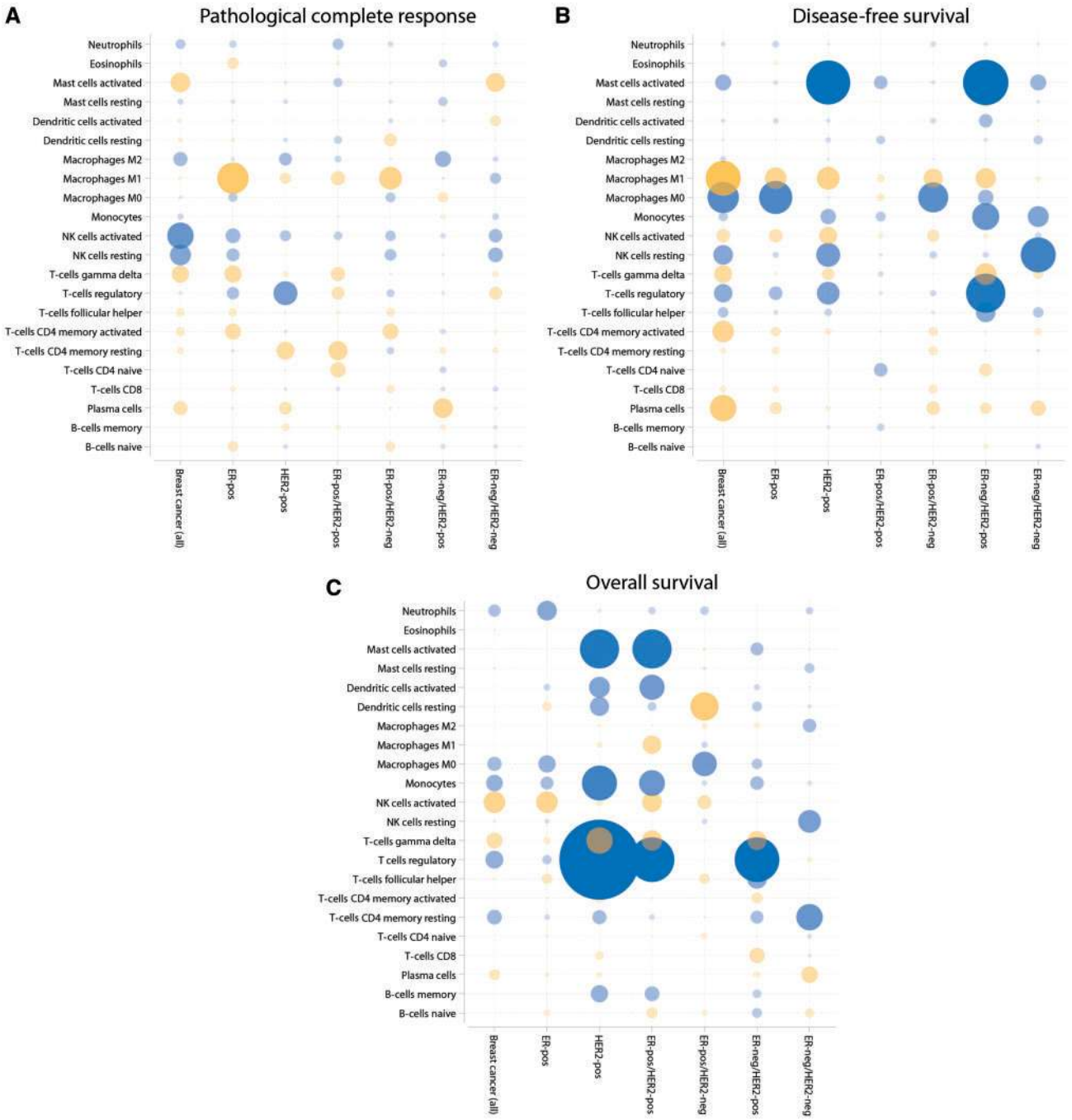

Figure 3. Bubble heat map for the predictive and prognostic values of immune cell–type fractions in breast cancer subtypes. Associations between fractions and **(A)** pathological complete response (pCR), **(B)** disease-free survival (DFS), and **(C)** overall survival (OS) were analyzed. A **blue bubble** indicates that a higher fraction is associated with lower pCR rate, shorter DFS, or shorter OS; a **yellow bubble** indicates that a higher fraction is associated with higher pCR rate, prolonged DFS, or prolonged OS. The **size of the bubble** indicates the statistical significance level. The predictive value of immune cell–type fractions in the neoadjuvant setting was assessed by multivariable binary logistic regression using pCR as outcome variable and age, T-stage, grade, lymph node involvement, estrogen receptor (ER) status, human epidermal growth factor receptor 2 (HER2) status, and treatment regimen as covariates. The prognostic value of immune cell–type fractions in the neoadjuvant and adjuvant settings was assessed by multivariable Cox regression analysis, with time to distant metastasis and time to death as outcome variables and age, tumor size, grade, lymph node involvement, ER status, HER2 status, and treatment regimen as covariates. DFS = disease-free survival; ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; NK = natural killer; OS = overall survival; pCR = pathological complete response; TNBC = triple-negative breast cancer.

status) with a higher pCR rate (OR = 1.68, 95% CI = 1.05 to 2.71), prolonged DFS (HR = 0.42, 95% CI = 0.29 to 0.61), and prolonged OS (HR = 0.49, 95% CI = 0.33 to 0.73) in comparison with the other three signatures. This applies to almost all subtypes based on receptor status. A high CD8+ T-cell exhaustion signature score was associated with shorter DFS in patients with ER-positive disease regardless of HER2 status (HR = 1.80, 95% CI = 1.07 to 3.04).

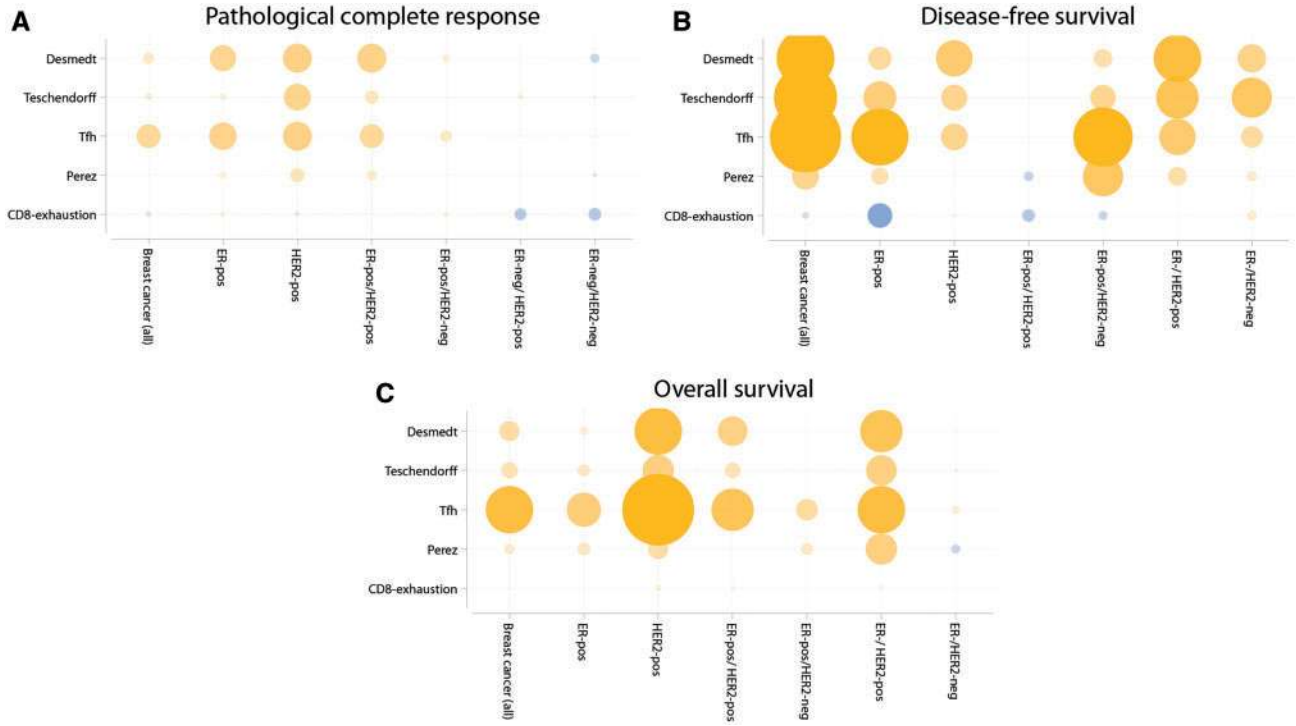

Figure 4. Bubble heat map for the predictive and prognostic values of immune gene signatures in breast cancer subtypes. Associations between fractions and **(A)** pathologicaly complete response (pCR), **(B)** disease-free survival (DFS), and **(C)** overall survival (OS) were analyzed. Signatures identified by Desmedt et al. (14), Teschendorff et al. (15), Perez et al. (16), Gu-Trantien et al. (Tfh signature) (17), and a CD8+ T-cell exhaustion signature (18) were investigated. A **blue bubble** indicates that a higher fraction is associated with lower pCR rate, shorter DFS, or shorter OS; a **yellow bubble** indicates that a higher fraction is associated with higher pCR rate, prolonged DFS, or prolonged OS. The **size of the bubble** indicates the statistical significance level. DFS = disease-free survival; ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; NK = natural killer; OS = overall survival, pCR = pathological complete response; TNBC = triple-negative breast cancer.

Discussion

We investigated the independent predictive and prognostic value of several in silico immune phenotypes in a large set of breast cancer patients. In our analyses, we included the clinicopathological parameters that are currently used in the clinical decision-making for neoadjuvant and adjuvant treatment. This provided insight into multiple immune parameters and their potential relevance for breast cancer management. This is of particular interest in light of the current clinical developments of immune-modulating therapies. Previously, it was thought that breast cancer was not an immunogenic cancer type, in contrast to melanoma or renal cell cancer. However, our unbiased approach suggests the hypothesis that the immune system is indeed involved in breast cancer. More specifically, our data indicate that specific immune cells, depending on breast cancer subtypes, are associated with highly relevant measures such as treatment response and survival.

First, we observed differences in subtypes with regard to immune cell fractions associated with response to neoadjuvant chemotherapy and survival. An estimated high regulatory T-cell fraction was associated with a lower pCR rate, as well as shortened DFS and OS, particularly in patients with HER2-positive breast cancers, irrespective of ER status. Previous studies have reported conflicting results regarding the prognostic value of regulatory T-cell infiltration for OS and DFS in breast cancer patients. These studies were either smaller, with 93 to 237 patients, or took a lower number of covariates into account in their analyses (33–37). These associations of high regulatory T-cell fraction with worse disease outcome parameters are of interest in the light of possible intervention strategies. For instance, the anti-CTLA-4 antibody ipilimumab has been shown to downregulate regulatory T-cell tumor infiltration in both melanoma and early-stage breast cancer (38,39).

A higher estimated $\gamma\delta$ T-cell fraction was associated with a higher pCR rate, especially in patients with ER-positive breast cancer, irrespective of HER2 status. In addition, in patients with HER2-positive/ER-negative tumors, a high $\gamma\delta$ T-cell fraction was associated with a prolonged DFS and OS. This is in line with recent findings from Gentles et al. (30), who reported that $\gamma\delta$ T-cells are the most statistically significant favorable prognostic immune cell population for 39 malignancies, including breast cancer. However, in that study no analysis of breast cancer subtypes was conducted, and fewer covariates were included to assess the independent prognostic value.

A high estimated M1 macrophage fraction was associated with a higher pCR rate in patients with ER-positive breast cancer (irrespective of HER2 status) and prolonged OS particularly in patients with ER-positive disease. This supports the current hypothesis that these macrophages are tumoricidal and therefore beneficial for prognosis (40). TAMs were previously associated with shorter survival in breast cancer patients (11–13), which has been attributed to their polarization towards the M2 subtype (41). In our analysis, however, we did not find an association between M2 macrophage fraction and response to neoadjuvant therapy, DFS, or OS. In contrast to M1 macrophages, a higher estimated fraction of M0 macrophages was associated with poor DFS, as well as shortened OS in patients with ER-positive breast cancer. These macrophages are formed from monocytes when entering the tissue and are not yet polarized

toward either the M1 or M2 macrophage subtypes. The hypothesis that M0 macrophage fraction seems relevant in both OS and DFS underlines its possible impact on intrinsic ER-positive breast cancer biology and deserves further attention in future studies. These apparently varying associations of macrophage subpopulations with disease outcome parameters is of great interest, particularly in light of the development of interventions affecting monocytes and macrophages (42).

In patients with TNBCs, we observed that a higher fraction of activated mast cells was associated with a higher pCR rate. This is in accordance with several studies in breast cancer that have linked mast cells to a good prognosis (43–46). However, in the present study, an increased fraction of activated mast cells was also associated with poor DFS and OS in patients with HER2-positive breast cancer. Indeed, mast cells are hypothesized to possess both antitumoral and protumoral properties (47), which might vary according to breast cancer subtype.

In patients with TNBC or HER2-positive breast cancer, we found that a higher fraction of resting NK cells was associated with worse DFS and OS. Interestingly, NK cells have the capacity to inhibit cytotoxic T-cell responses in mice and humans (48). The association with worse DFS is in line with the lower pCR rate we observed for a higher fraction of NK cells (resting and activated) for patients with breast cancer in general. The role of NK cells in the clinical outcome in TNBC may provide for a future therapeutic target in TNBC.

With regard to functionality of immune cells in breast cancer, our data suggest that a high score on the McKinney signature for CD8+ T-cell exhaustion (18) is associated with poor DFS in patients with ER-positive breast cancer. The relevance of T-cell exhaustion in breast cancer, particularly in light of its apparent subtype relatedness, has hardly been considered in previous studies. In chronic viral infection, CD8+ T-cell exhaustion has recently been related to poor outcome (49), indicating its relation to immune system evasion. In addition, Poschke et al. reported signs of exhaustion, such as loss of CD28, on tumor-associated as compared with blood-derived CD8+ T-cells in early-stage breast cancer (50). Together with our results, these data suggest the hypothesis that CD8+ T-cell exhaustion is also related to tumor immune evasion in breast cancer.

As we consider this simple pooled analysis as hypothesis-generating to gain insight into which immune cell–type fractions and signatures could be of interest as independent predictive or prognostic factors, we wanted to keep the power to detect potentially relevant signals as high as possible (ie, lower type II error). Therefore, we chose not to pursue a split-sample approach with a discovery and validation cohort, which would decrease the type I error (ie, false-positive findings). We think that any future use of immune cell–type fractions and signature as independent predictive and prognostic factors in breast cancer management warrants additional validation in well-designed studies controlling the type I error.

The main hypothesis generated in our unbiased in silico approach is that a multitude of immune cells are related to treatment response and outcome in breast cancer. Varying immune cell fractions seem to be important in particular breast cancer subtypes, indicating the complexity of immune system involvement in breast cancer. The results of our study also justify an unbiased approach for gaining insight into this system. The recent study by Nanda et al. has provided initial indications that immunotherapy can be effective for treating breast cancer (51). Even in ER-positive breast cancer, which was previously considered a particularly nonimmunogenic disease, preliminary data have shown clinical efficacy of immunotherapy (52). However, as in TNBC, this was the case only in a subset of patients. Insight into how to select the best treatment for the right patient is urgently needed. The present study may provide a further step in that direction.

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Notes

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CPS and RSNF were responsible for the conception and design of this study. RDB and RSNF collected and assembled data. All authors contributed to data analysis and interpretation, the writing of this manuscript, and the final decision to submit the manuscript.

References

1. Harris LN, You F, Schnitt SJ, et al. Predictors of resistance to preoperative trastuzumab and vinorelbine for HER2-positive early breast cancer. *Clin Cancer Res*. 2007;13(4):1198–1207.
2. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet*. 2011; 378(9793):771–784.
3. Emens L, Braiteh F, Cassier P, et al. Inhibition of PD-L1 by MPDL3280A leads to clinical activity in patients with metastatic triple-negative breast cancer. *San Antonio Breast Cancer Symposium* 2014;abstr PD1-6.
4. Nanda R, Chow L, Dees E, et al. A phase Ib study of pembrolizumab (MK-3475) in patients with advanced triple-negative breast cancer. *San Antonio Breast Cancer Symposium* 2014;abstr S1-09.
5. Loi S, Sirtaine N, Piette 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):860–867.
6. Loi S, Michiels S, Salgado R, 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): 1544–1550.
7. Salgado R, Denkert C, Campbell 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. *JAMA Oncol*. 2015;1(4):448–454.
8. Denkert C, Loibl S, Noske A, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol*. 2010;28(1):105–113.
9. Ono M, Tsuda H, Shimizu C, et al. Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer. *Breast Cancer Res Treat*. 2012;132(3):793–805.
10. Denkert C, von Minckwitz G, Brase JC, 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):983–991.
11. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast *cancer*. *Cancer Res*. 1996;56(20):4625–4629.
12. Campbell MJ, Tonlaar NY, Garwood ER, et al. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast Cancer Res Treat*. 2011;128(3):703–711.
13. Zhang Y, Cheng S, Zhang M, et al. High-infiltration of tumor-associated macrophages predicts unfavorable clinical outcome for node-negative breast cancer. *PLoS One*. 2013;8(9):1–8.

14. Desmedt C, Haibe-Kains B, Wirapati P, et al. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. *Clin Cancer Res*. 2008;14(16):5158–5165.
15. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol*. 2007;8(8):R157.
16. Perez EA, Thompson EA, Ballman KV, et al. Genomic analysis reveals that immune function genes are strongly linked to clinical outcome in the North Central Cancer Treatment Group N9831 Adjuvant Trastuzumab Trial. *J Clin Oncol*. 2015;33(7):701–708.
17. Gu-Trantien C, Loi S, Garaud S, et al. CD4+ follicular helper T-cell infiltration predicts breast cancer survival. *J Clin Invest*. 2013;123(7):1–20.
18. McKinney EF, Lyons PA, Carr EJ, et al. A CD8+ T-cell transcription signature predicts prognosis in autoimmune disease. *Nat Med*. 2010;16(5):586–591.
19. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods*. 2015;12(5):453–457.
20. Barrett T, Wilhite SE, Ledoux P, et al. NCBI GEO: Archive for functional genomics data sets - update. *Nucleic Acids Res*. 2013;41(D1):991–995.
21. Crijns APG, Fehrmann RSN, De Jong S, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. *PLoS Med*. 2009;6(2):0181–0193.
22. Heijink DM, Fehrmann RSN, de Vries EGE, et al. A bioinformatical and functional approach to identify novel strategies for chemoprevention of colorectal cancer. *Oncogene*. 2011;30(17):2026–2036.
23. Fehrmann RSN, Karjalainen JM, Krajewska M, et al. Gene expression analysis identifies global gene dosage sensitivity in cancer. *Nat Genet*. 2015;47(2):115–125.
24. Hammond MEH, Hayes DF, Dowsett M, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J Clin Oncol*. 2010;28(16):2784–2795.
25. Wolff AC, Hammond MEH, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *J Clin Oncol*. 2013;31(31):3997–4013.
26. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*. 2003;100(14):8418–8423.
27. Parker JS, Mullins M, Cheung MCU, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol*. 2009;27(8):1160–1167.
28. Hu Z, Fan C, Oh DS, et al. The molecular portraits of breast tumors are conserved across microarray platforms. *BMC Genomics*. 2006;7:96.
29. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011;121(7):2750–2767.
30. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. 2015;21(8):1–12.
31. Ignatiadis M, Singhal SK, Desmedt C, et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J Clin Oncol*. 2012;30(16):1996–2004.
32. Korn EL, Troendle JF, McShane LM, Simon R. Controlling the number of false discoveries: application to high-dimensional genomic data. *J Stat Plan Inference*. 2014;124(2):379–398.
33. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T-cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol*. 2006;24(34):5373–5380.
34. Liu F, Lang R, Zhao J, et al. CD8 + cytotoxic T-cell and FOXP3 + regulatory T-cell infiltration in relation to breast cancer survival and molecular subtypes. *Breast Cancer Res Treat*. 2011;130(2):645–655.
35. Liu S, Foulkes WD, Leung S, et al. Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. *Breast Cancer Res*. 2014;16(5):1–12.
36. Merlo A, Casalini P, Carcangiu ML, et al. FOXP3 expression and overall survival in breast cancer. *J Clin Oncol*. 2009;27(11):1746–1752.
37. Aruga T, Suzuki E, Saji S, et al. A low number of tumor-infiltrating FOXP3-positive cells during primary systemic chemotherapy correlates with favourable anti-tumor response in patients with breast cancer. *Oncol Rep*. 2011;25(1):223–230.
38. Romano E, Kusio-Kobialka M, Foukas PG, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T-cells ex vivo by nonclassical monocytes in melanoma patients. *Proc Natl Acad Sci U S A*. 2015;112(19):6140–6145.
39. Diab A, McArthur H, Solomon S, Sacchin I, Comstock C, Maybody M. A pilot study of preoperative (Pre-op), single-dose ipilimumab (Ipi) and/or cryoablation (Cryo) in women (pts) with early-stage/resectable breast cancer. *J Clin Oncol*. 2014;32;5s(suppl; abstr 1098).
40. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11):549–555.
41. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. 2010;22(2):231–237.
42. Nywening TM, Wang-Gillam A, Sanford DE, et al. Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial. *Lancet Oncol*. 2016;17(5):651–662.
43. Aaltomaa S, Lipponen P, Palpinaho S, Kosma V. Mast cells in breast cancer. *Anticancer Res*. 1993;13(3):785–788.
44. Dabiri S, Huntsman D, Makretsov N, et al. The presence of stromal mast cells identifies a subset of invasive breast cancers with a favorable prognosis. *Mod Pathol*. 2004;17(6):690–695.
45. Rajput A, Turbin D, Cheang M, et al. Stromal mast cells in invasive breast cancer are a marker of favourable prognosis: A study of 4,444 cases. *Breast Cancer Res Treat*. 2008;107(2):249–257.
46. Rovere F Della, Granata A, Familiari D, D'Arrigo G, Mondello B, Basile G. Mast cells in invasive ductal breast cancer: Different behavior in high and minimum hormone-receptive cancers. *Anticancer Res*. 2007;27(4B):2465–2471.
47. Khazaie K, Blatner NR, Khan MW, et al. The significant role of mast cells in cancer. *Cancer Metastasis Rev*. 2011;30(1):45–60.
48. Crome S, Lang P, Lang K, Ohashi P. Natural killer cells regulate diverse T-cell responses. *Trends Immunol*. 2013;34(7):342–349.
49. McKinney EF, Lee JC, Jayne DRW, Lyons PA, Smith KGC. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature*. 2015;523(7562):612–616.
50. Poschke I, De Boniface J, Mao Y, Kiessling R. Tumor-induced changes in the phenotype of blood-derived and tumor-associated T-cells of early stage breast cancer patients. *Int J Cancer*. 2012;131(7):1611–1620.
51. Nanda R, Chow LQM, Dees EC, et al. Pembrolizumab in patients with advanced triple-negative breast cancer: Phase Ib KEYNOTE-012 study. *J Clin Oncol*. 2016; in press.
52. Rugo H, Delord J-P, Im S-A, et al. Preliminary efficacy and safety of pembrolizumab (MK-3475) in patients with PD-L1–positive, estrogen receptor-positive (ER+)/HER2-negative advanced breast cancer enrolled in KEYNOTE-028. *San Antonio Breast Cancer Symposium* 2015;abstr S5-S07.