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ARTICLE

Immune Infiltration in Invasive Lobular Breast Cancer

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Abstract

Background: Invasive lobular breast cancer (ILC) is the second most common histological subtype of breast cancer after invasive ductal cancer (IDC). Here, we aimed at evaluating the prevalence, levels, and composition of tumor-infiltrating lymphocytes (TILs) and their association with clinico-pathological and outcome variables in ILC, and to compare them with IDC.

Methods: We considered two patient series with TIL data: a multicentric retrospective series ($n = 614$) and the BIG 02-98 study ($n = 149$ ILC and 807 IDC). We compared immune subsets identified by immuno-histochemistry in the ILC ($n = 159$) and IDC ($n = 468$) patients from the Nottingham series, as well as the CIBERSORT immune profiling of the ILC ($n = 98$) and IDC ($n = 388$) METABRIC and The Cancer Genome Atlas patients. All ILC/IDC comparisons were done in estrogen receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative tumors. All statistical tests were two-sided.

Results: TIL levels were statistically significantly lower in ILC compared with IDC (fold-change = 0.79, 95% confidence interval = 0.70 to 0.88, $P < .001$). In ILC, high TIL levels were associated with young age, lymph node involvement, and high proliferative tumors. In the univariate analysis, high TIL levels were associated with worse prognosis in the retrospective and BIG 02-98 lobular series, although they did not reach statistical significance in the latter. The Nottingham series revealed that the levels of intratumoral but not total CD8⁺ were statistically significantly lower in ILC compared with IDC. Comparison of the CIBERSORT profiles highlighted statistically significant differences in terms of immune composition.

Conclusions: This study shows differences between the immune infiltrates of ER-positive/HER2-negative ILC and IDC in terms of prevalence, levels, localization, composition, and clinical associations.

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The presence and potential clinical value of tumor-infiltrating lymphocytes (TILs) have been assessed in many studies. Increased lymphocytic infiltration has been shown to be more frequent in triple-negative and human epidermal growth factor receptor 2 (HER2)-positive breast cancer and to be associated with an increased pathological complete response rate after neo-adjuvant chemotherapy, as well as with improved survival after adjuvant chemotherapy in these subtypes (1–3). Given that various methods have been used to quantify TILs in breast cancer, efforts to standardize their assessment were undertaken, resulting in the first guidelines for scoring TILs (4). A recent publication also showed that the assessment of stromal TILs, those present in the tumor stromal tissue, is more reproducible than intratumoral TIL evaluation (5). A complementary approach to evaluate immune infiltrates is to apply a deconvolution algorithm to gene expression data, such as CIBERSORT, which estimates the relative proportion of various immune cell types (6–8).

Invasive lobular breast cancer (ILC) represents the second most common histological subtype in breast cancer after invasive breast carcinoma of no special type (NST), previously known and further referred to as invasive ductal carcinoma (IDC), and accounts for up to 15% of cases (9). These tumors generally express estrogen receptor (ER) and lack HER2 amplification. Clinically, patients with ILC differ from those with IDC. They tend to relapse later and present a different metastatic pattern (9,10). Recently, The Cancer Genome Atlas (TCGA), the RATHER consortium, METABRIC, and our group characterized the genomic landscape of a large number of ILC tumors and consistently demonstrated that ILC presents differences regarding the frequency of alterations in several cancer genes compared with IDC (11–14). Of potential relevance, TCGA and RATHER consortia also identified an “immune-related” ILC transcriptomic subtype. However, when comparing these immune subtypes on the same data sets, it seems that they were not identifying the same tumors, suggesting that their definition should be further investigated (11,12).

While TILs in breast cancer continue to attract considerable attention, to our knowledge, there are no previous reports interrogating TILs in large cohorts of ILC. Using our multicentric retrospective cohort of ILC (13), we aimed to evaluate the distribution of TILs, correlate TIL levels with standard clinico-pathological parameters, investigate their prognostic value, and explore associations between TILs and transcriptomic features and recurrent genomic alterations in ILC. We further aimed to compare TILs between ER-positive/HER2-negative ILC and IDC tumors in the BIG 02-98 study (15). Finally, going beyond TILs to further enhance our understanding of the immune infiltrates in ER-positive/HER2-negative ILC and IDC tumors, we compared the proportion and localization of several immune subsets in the Nottingham series (16–18) and the estimates of the proportion of the different immune cell types provided by CIBERSORT in the METABRIC and TCGA cohorts (7,11,19).

Methods

Patients and Samples

The retrospective multicentric series of primary ILC has already been described (13). [Supplementary Tables 1 and 2](#) (available online) provide overview and patient-level information, respectively, of the main clinical characteristics while [Supplementary Figure 1](#) (available online) reports the number of patients and

samples considered for each analysis. The comparison of TILs in terms of prevalence and clinico-pathological associations between ER-positive/HER2-negative ILC ($n = 149$) and IDC ($n = 807$) was assessed in the BIG 02-98 study ([Supplementary Table 3](#), available online) (15). From this study, we only considered those patients whose tumor samples underwent central revision for ER and HER2 (20). For the Nottingham series, we only considered the ER-positive/HER2-negative ILC ($n = 159$) and IDC ($n = 468$) cases (16–18). The patient and tumor characteristics from all investigated ER-positive/HER2-negative ILC and IDC cases are reported in [Supplementary Table 4](#) (available online). This project has been approved by the ethics committee of the Institut Jules Bordet (CE1726 and CE2560).

Assessment of Immune Infiltration

Stromal TIL, the percentage of tumor stromal area containing TILs and hereinafter referred to as “TIL,” was independently evaluated by three pathologists (GVE, GP, and RS) on hematoxylin and eosin-stained slides of the retrospective ILC cohort ($n = 614$) using the protocols described in Salgado et al. (4). To evaluate the reliability of TIL assessments, the two-way random intraclass correlation coefficient (ICC) was estimated (21). All cases for which the discordance between at least two pathologists reached greater than 15% were first independently re-scored by all three pathologists and then reviewed together until a consensus was reached. For each pathologist, consensus values were used to recalibrate the other measurements through Passing-Bablok regression (22). The log transformation was applied to TIL values in order to stabilize variance and achieve an approximately symmetric distribution according to the measurement process (23). In the analyses, the arithmetic mean over the three pathologists of the log values was used. In the BIG 02-98 study, TILs were assessed previously by two pathologists, including RS, using the same method and the results described in Loi et al. (15). For this study, we considered the arithmetic mean of the log values from the two pathologists.

Statistical Analysis

Univariate and multiple linear regression models of log-transformed TIL percentage vs clinico-pathological and genomic variables were fitted. Point estimates and 95% confidence intervals of fold-change of TIL percentage were obtained. In the retrospective ILC cohort and the BIG2-98 series, we used the previously reported survival end points, namely breast cancer-free interval (BCFI) (13) and disease-free survival (DFS) (15), respectively. The association between the survival end points and the immune variables was evaluated using a Cox regression model by considering TILs as either continuous or categorical values, with or without adjustment for clinico-pathological variables. Statistical significance was assessed using the Wald test. For the comparison of the various immune markers in the Nottingham series, we resorted to the proportional odds logistic model extension of the Mann-Whitney test according to Harrell (24) to adjust for histology, age, grade, axillary lymph node status, and Ki67. CIBERSORT data from the METABRIC and TCGA series were obtained from Ali et al. ($n = 98$ ILC and $n = 388$ IDC) (7) and compared using the Wilcoxon rank sum test. All statistical tests were two-sided, and P values of less than .05 were considered statistically significant. Whenever appropriate, we applied correction for multiple testing using the

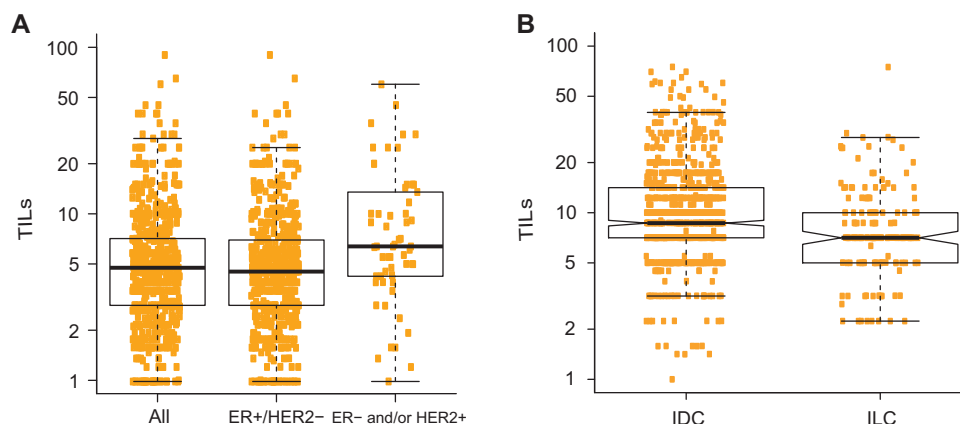


Figure 1. Distribution of tumor-infiltrating lymphocytes (TILs). Histograms displaying the distribution of the TIL counts in all invasive lobular carcinoma (ILC) cases, and only in the estrogen receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative ones (A), as well as the distribution of ER-positive/HER2-negative ILC and invasive ductal carcinoma cases from the BIG 02-98 study (B). ER = estrogen receptor; IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; TIL = tumor-infiltrating lymphocyte.

Benjamini-Hochberg method (25). All analyses were performed using R v3.4.1 (26).

Results

Distribution of TILs

The intraclass correlation coefficient between the three pathologists for TIL evaluation on the 614 ILC cases from the multicentric retrospective series was 0.71 (95% confidence interval [CI] = 0.65 to 0.76). ILC was characterized by low lymphocytic infiltration, with a median percentage of TILs of 5% and an interquartile range of 3% to 7% (Figure 1A). Fifteen percent of the samples had more than 10% of TIL. We compared the distribution of TILs in ER-positive/HER2-negative ILC with ER-positive/HER2-negative IDC tumors from the BIG 02-98 study (15) and observed that TIL counts were slightly but statistically significantly lower in ILC compared with IDC (fold-change = 0.79, 95% CI = 0.70 to 0.88, $P < .001$) (Figure 1B).

Association Between TILs and Clinico-Pathological Variables

We investigated the association between TILs as a continuous variable and the standard clinico-pathological variables. In the retrospective series, we observed at the univariate level that higher TIL values were associated with ER- and PR-negative status, high proliferation status ($\geq 20\%$ Ki67), high grade, node-positive status, and young age at diagnosis (Figure 2A; Supplementary Table 5, available online). We further observed a statistically significant increase and decrease of TILs for the mixed nonclassic and the alveolar subtypes, respectively, compared with the more common classic subtype. In the multivariable regression, a similar trend was detected for these variables, with the exception of tumor grade, for which the association was no longer statistically significant. The associations between TILs and age, as well as Ki67 in all ILC patients from BIG 02-98 ($n = 204$), were consistent with our results (Supplementary Figure 2 and Supplementary Table 6, available online). The association with lymph node involvement could not be investigated as all patients included in this trial had positive lymph nodes. When restricting the analyses to the

ER-positive/HER2-positive ILC in our cohort ($n = 555$), we made similar observations as in the global ILC population regarding the association with high Ki67, node-positive status, and young age at diagnosis (Figure 2A; Supplementary Table 7, available online). We next used the TIL data collected in the BIG 02-98 trial to compare the associations in ER-positive/HER2-negative IDC ($n = 807$) and ILC ($n = 149$). TILs were positively associated with Ki67 both in IDC and ILC. However, the associations with age and with grade were restricted to ILC and IDC, respectively (Figure 2B; Supplementary Tables 8 and 9, available online).

Association Between TILs and Genomic Alterations

Genomic alterations with oncogenic potential (substitutions and indels as previously defined (27) were available for 399 patients of the multicentric retrospective ILC series (13), leading us to evaluate the association between TILs and the mutational status of the 15 genes that were mutated in at least 2% of the tumors. We observed statistically significantly lower TIL counts in tumors harboring *ERBB3* mutations and higher TIL counts in tumors presenting somatic oncogenic *TP53*, *ARID1A*, and *BRCA2* mutations compared with tumors that were not mutated in those genes. *KMT2C* and *PIK3CA* mutated tumors were associated with higher TIL, although not statistically significantly (Figure 3A; Supplementary Table 10, available online). All associations remained statistically significant in multivariable models with standard clinico-pathological variables, with the exception of *BRCA2*. We further investigated the association between TILs and copy number aberrations (CNAs) present in at least 5% of the patients. Genome-wide copy number data were available for 166 cases. We observed statistically significantly higher TIL values in tumors with 8q24.23 loss (which included *PTK2*) and 7p21.2 gains (*ETV1*), but statistically significantly fewer TILs in tumors with 5q gains (Figure 3B; Supplementary Table 11, available online).

Association Between TILs and Transcriptomic Features

In order to better understand the transcriptomic phenotype associated with infiltrated ILC tumors, we compared the gene expression profiles between ILC tumors with low ($\leq 5\%$) and high TIL levels ($> 10\%$), microarray gene expression data being

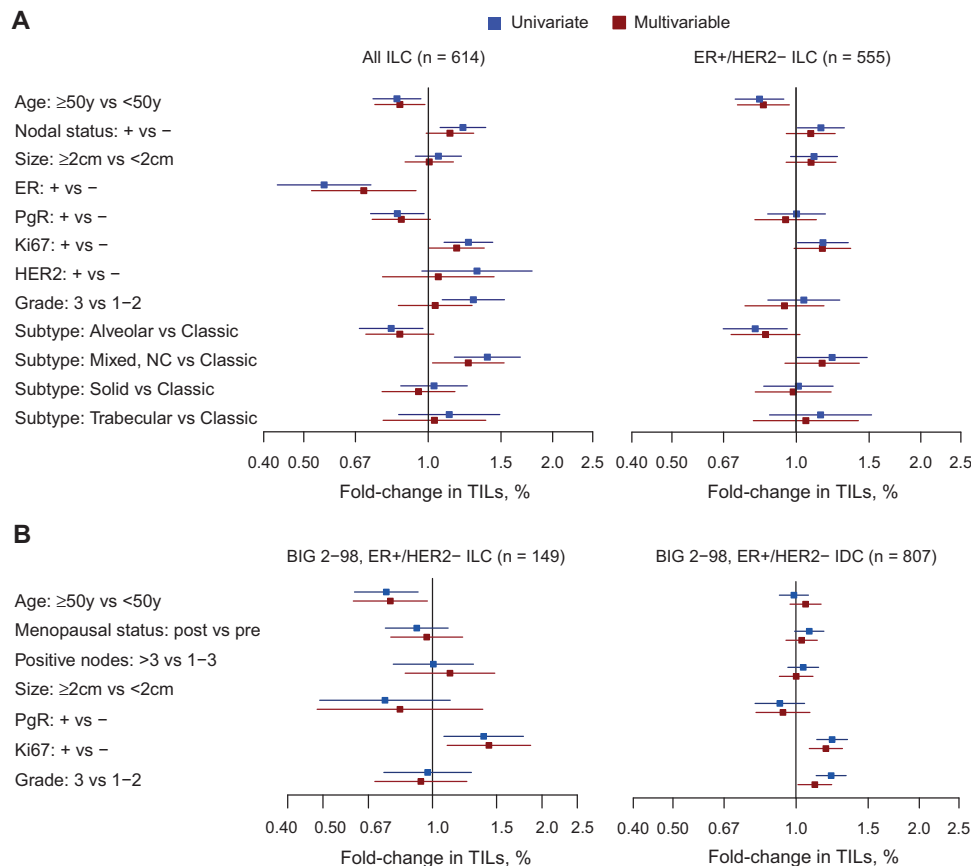


Figure 2. Associations between tumor-infiltrating lymphocytes and clinico-pathological variables. Univariate and multivariable associations between tumor-infiltrating lymphocyte and clinico-pathological variables in all invasive lobular carcinoma (ILC) cases and only the estrogen receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative from our series (A), as well as from the ER-positive/HER2-negative ILC and invasive ductal carcinoma (B) from the BIG 02-98 study. ER = estrogen receptor; IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; TIL = tumor-infiltrating lymphocyte.

available for 117 tumors of the multicentric ILC cohort. Thirty-three genes were overexpressed in the high-TIL group with a fold-change of 1.2 or greater compared with the low-TIL group, and a corrected *P* value of .05 or less. This list included, among others, genes involved in cellular defense response (GO:0006968; *ITK*, *CXCL9*, *TRAT1*, *SH2D1A*, and *CCR6*) and regulation of antigen receptor-mediated signaling pathway (GO:0050854; *PRKCB*, *TRAT1*, *CCR7*, *UBASH3A*) (Figure 3C). We then computed a score based on these 33 genes and compared it with existing immune and other gene expression signature scores (28–37). We observed a strong correlation with several previously reported immune expression signatures (Figure 3D), suggesting that the immune infiltrates present in ILC might share at least some similarities with the ones found in the global breast cancer population.

Comparison of the Prevalence and Localization of Different Immune Cell Types Between ER-Positive/HER2-Negative ILC and IDC

We further aimed at extending our immune comparison between ER-positive/HER2-negative ILC and IDC beyond TIL counts by interrogating immuno-histochemical data on various immune markers according to their localization (total staining or staining restricted to the intratumoral, adjacent, or distant stromal compartments) in the Nottingham series (16–18), and the CIBERSORT immune profiles in the METABRIC and TCGA data sets (7).

We compared the scores for CD3⁺, CD8⁺, CD20⁺, CD68⁺, and FOXP3⁺ cells between the 159 ILC and 468 IDC samples from the Nottingham series (Figure 4A). After adjusting for standard clinico-pathological variables, we observed statistically significantly lower levels in ILC compared with IDC regarding the adjacent stromal CD3⁺, the intratumoral CD8⁺, all measures of CD68⁺, and all except the intratumoral measures of FOXP3⁺ cells. To complement this analysis, we compared the relative proportion of the various immune cell types recognized by CIBERSORT (7) and reported the statistically significant associations in Supplementary Figure 4 (available online). Importantly, as in Ali et al. (7), we considered only samples with a CIBERSORT *P* value of less than .05, excluding samples without immune infiltration, leaving 388 ER-positive/HER2-negative IDC samples to the 98 ER-positive/HER2-negative ILC samples. The comparison revealed a lower frequency of follicular helper and gamma delta T cells, as well as M0, M1, and to a lesser extent M2 macrophages in ILC compared with IDC, but a higher frequency of B memory cells, monocytes, and CD8⁺ T cells. Unfortunately, these results do not provide information regarding the localization of these different immune cell types as CIBERSORT derives the immune subtyping from bulk transcriptomic data.

Association Between TILs and Survival

Finally, we assessed the prognostic value of TILs in ILC. TILs were initially considered a continuous variable for survival

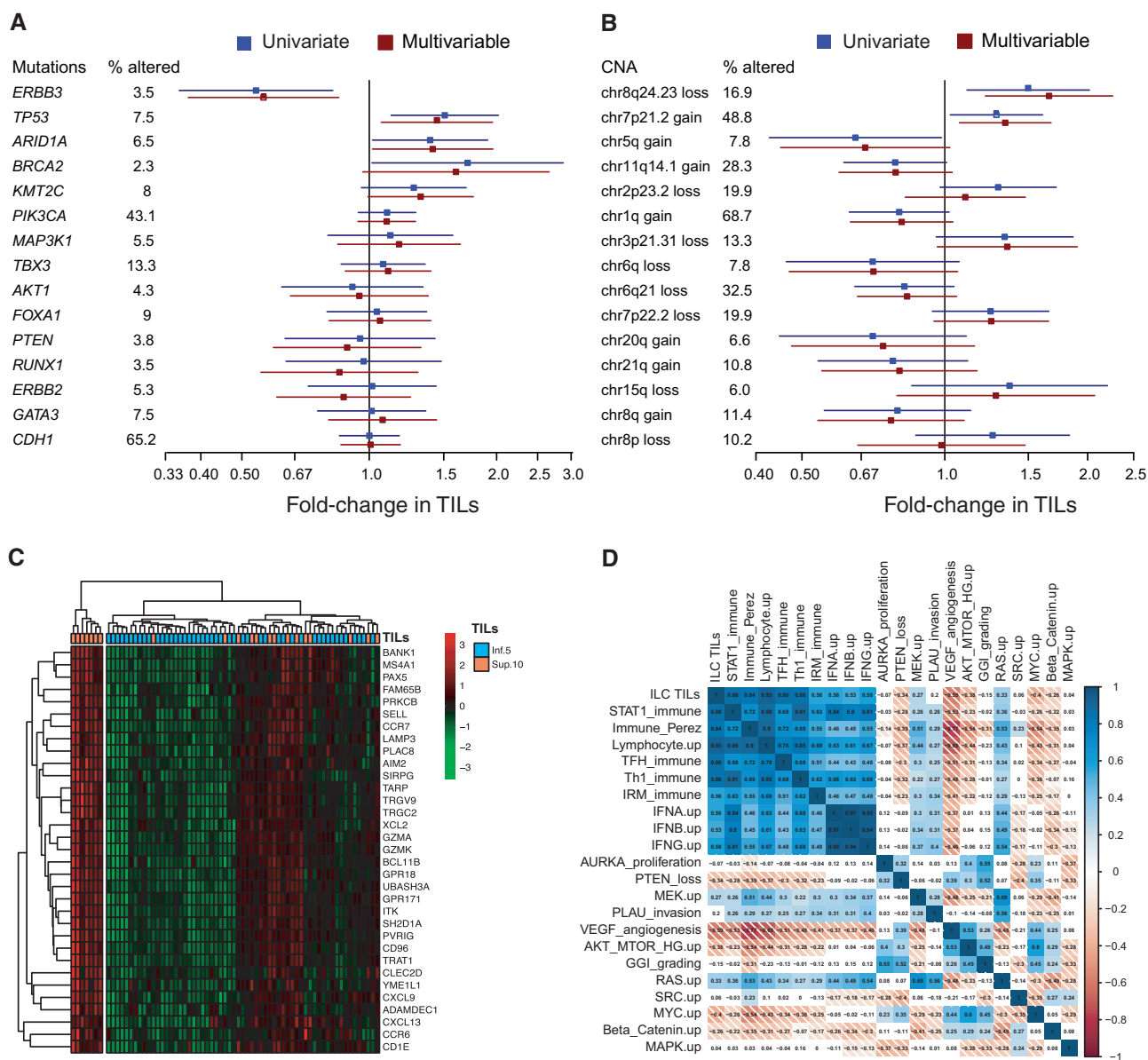


Figure 3. Associations between tumor-infiltrating lymphocytes (TILs), genomic and transcriptomic features in the retrospective invasive lobular carcinoma (ILC) cohort. Univariate and multivariable associations between TIL and recurrently mutated genes ($n = 399$) (A) or recurrent copy number aberrations ($n = 166$) (B). Heatmap of the genes statistically significantly associated with TILs at the transcriptomic level (C). Correlation matrix of the lobular immune gene expression signature (ILC TIL) with published immune signatures (D). CNA = copy number aberration; ILC = invasive lobular carcinoma; TIL = tumor-infiltrating lymphocyte.

analysis, revealing that higher TILs were associated with a worse prognosis in ILC in univariate (HR = 1.22 for each 10% increment, 95% CI = 1.06 to 1.41, $P = .005$) but not multivariable analysis in the retrospective cohort (Supplementary Table 12, available online). TILs were next considered a categorical variable using the following groups, with the cut-points adapted from the interquartile range rounded to the nearest multiple of 5: low ($\leq 5\%$), intermediate (>5 and $\leq 10\%$), and high ($>10\%$). Both the high and intermediate groups were associated with worse breast cancer-free interval (BCFI) compared with the lowest TIL values (HR_{high vs low} = 1.84, 95% CI = 1.22 to 2.78, $P = .004$; HR_{intermediate vs low} = 1.46, 95% CI = 1.02 to 2.09, $P = .04$) (Figure 5A). In multivariable analysis adjusting for the standard clinicopathological variables, the association between BCFI and TILs in the group with high TIL values lost statistical significance

whereas the association remained statistically significant for the group with intermediate TILs (Supplementary Table 13, available online). Exploratory analyses in the ER-positive/HER2-negative subgroup revealed a biologically consistent but statistically nonsignificant difference between the high and low groups (HR = 1.58, 95% CI = 0.98 to 2.54, $P = .06$) (Supplementary Table 14, available online), while no statistically significant difference was observed between the TIL groups in the ER-negative and/or HER2-positive subgroup (Figure 5B; Supplementary Figure 3A and Supplementary Table 15, available online). We further assessed the prognostic value of TILs in the BIG 02-98 series, and, similar to the retrospective cohort, we observed that higher TILs were associated with worse prognosis at the univariate level, although not reaching statistical significance (Figure 5C; Supplementary Table 16, available online). While the separation

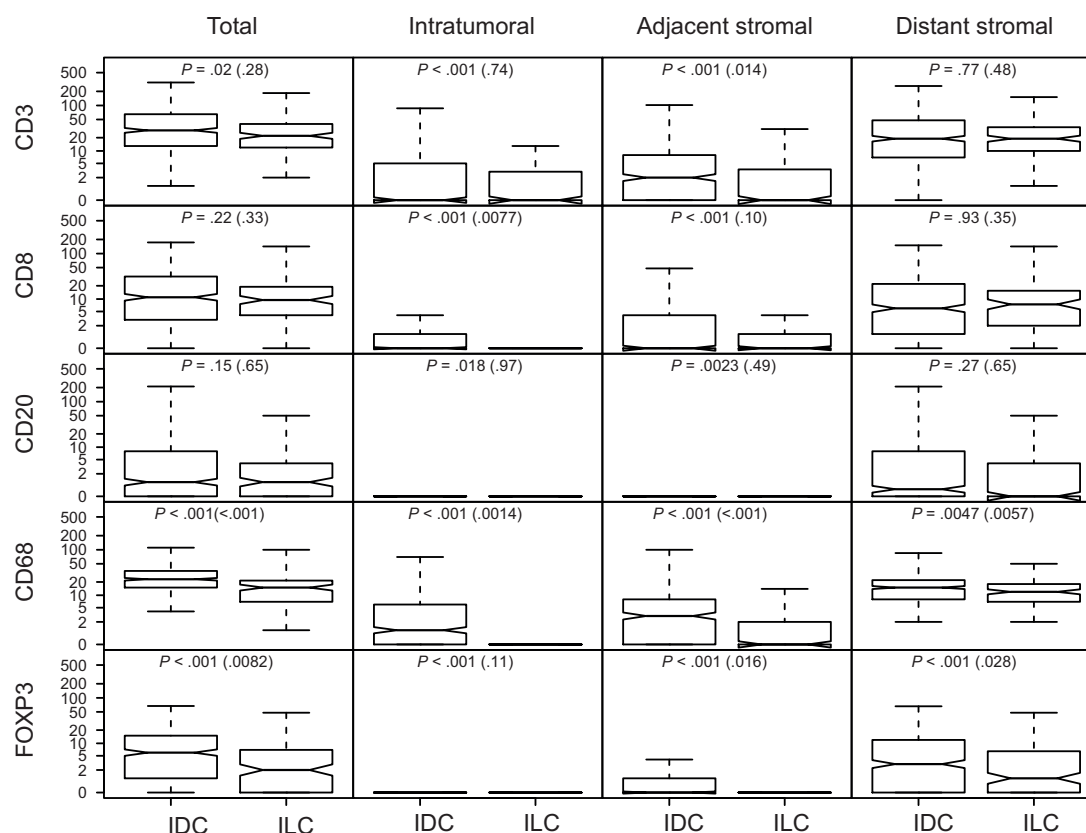


Figure 4. Comparison of the expression of various immune cell types between estrogen receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative invasive lobular carcinoma. Distribution of the expression of the various immune markers assessed by immunohistochemistry in the Nottingham cohort according to the histology and their localization. For each comparison, the value in parentheses refers to the multivariable-adjusted P value from the proportional odds logistic model extension of the Mann-Whitney test according to Harrell (24) with the following covariates: histological subtype, age, grade, axillary lymph node, and Ki67 status. ER = estrogen receptor; ILC = invasive lobular carcinoma.

of the curves was visible for the ER-positive/HER2-negative ILC subgroup (Figure 5D; Supplementary Table 17, available online), the curves were completely superimposed for the ER-positive/HER2-negative IDC (Supplementary Figure 3B and Supplementary Table 18, available online).

Discussion

This work represents to our knowledge the most detailed and comprehensive assessment of TILs using standardized, reproducible protocols and the largest series of primary ILC samples to date. We demonstrated that most ILCs are characterized by low levels of TILs. The vast majority of ILC tumors (~90%) are ER-positive/HER2-negative, making our findings consistent with previous reports showing that TILs are more prevalent in triple-negative and HER2-positive breast cancer (15,38,39). Droeser et al. reported lower levels of lymphocytic infiltrates in ILC compared with IDC, based on the percentage of CD4⁺ immunohistochemistry-stained cells; however, this comparison was flawed because they included all IDC and ILC tumors irrespective of ER and HER2 status (40). Our comparison of ER-positive/HER2-negative ILC and IDC in the BIG 02-98 study, however, confirmed the slight but statistically significant increase in TILs in IDC, even after adjusting for clinico-pathological variables.

Several important findings emerged when comparing the associations between TILs, clinico-pathological variables, and survival between ER-positive/HER2-negative ILC and IDC. First, while high TIL levels were associated with high proliferation status as defined by Ki67 in both histological subtypes, the association of young age and high grade with high TIL levels was only seen in ILC and IDC, respectively. Second, the survival analyses in our retrospective ILC cohort revealed that increased TILs were associated with worse BCFI at the univariate level. This was the case when TILs were assessed either as a continuous or categorical variable. This observation was somehow contrasting to what has been reported in triple-negative and HER2-positive breast cancer, where increased TILs were associated with better prognosis (1,2). However, in the multivariable analysis, TILs lost their prognostic value at the continuous level, although in the categorical analysis, the group with intermediate TIL values remained associated with worse prognosis compared with the group with low TILs. This loss of prognostic association, once adjusted for the standard clinico-pathological variables, might be explained by the association of TILs with poor prognostic features. Of interest, in the BIG 02-98 study, we observed at the univariate level that high TILs were associated with worse prognosis in ILC, although without reaching statistical significance. Interestingly, in this study, TILs did not show any association with survival in ER-positive/HER2-negative IDC.

When we compared the immuno-histochemical scores (total, intratumoral, adjacent stromal, and distant stromal) of the

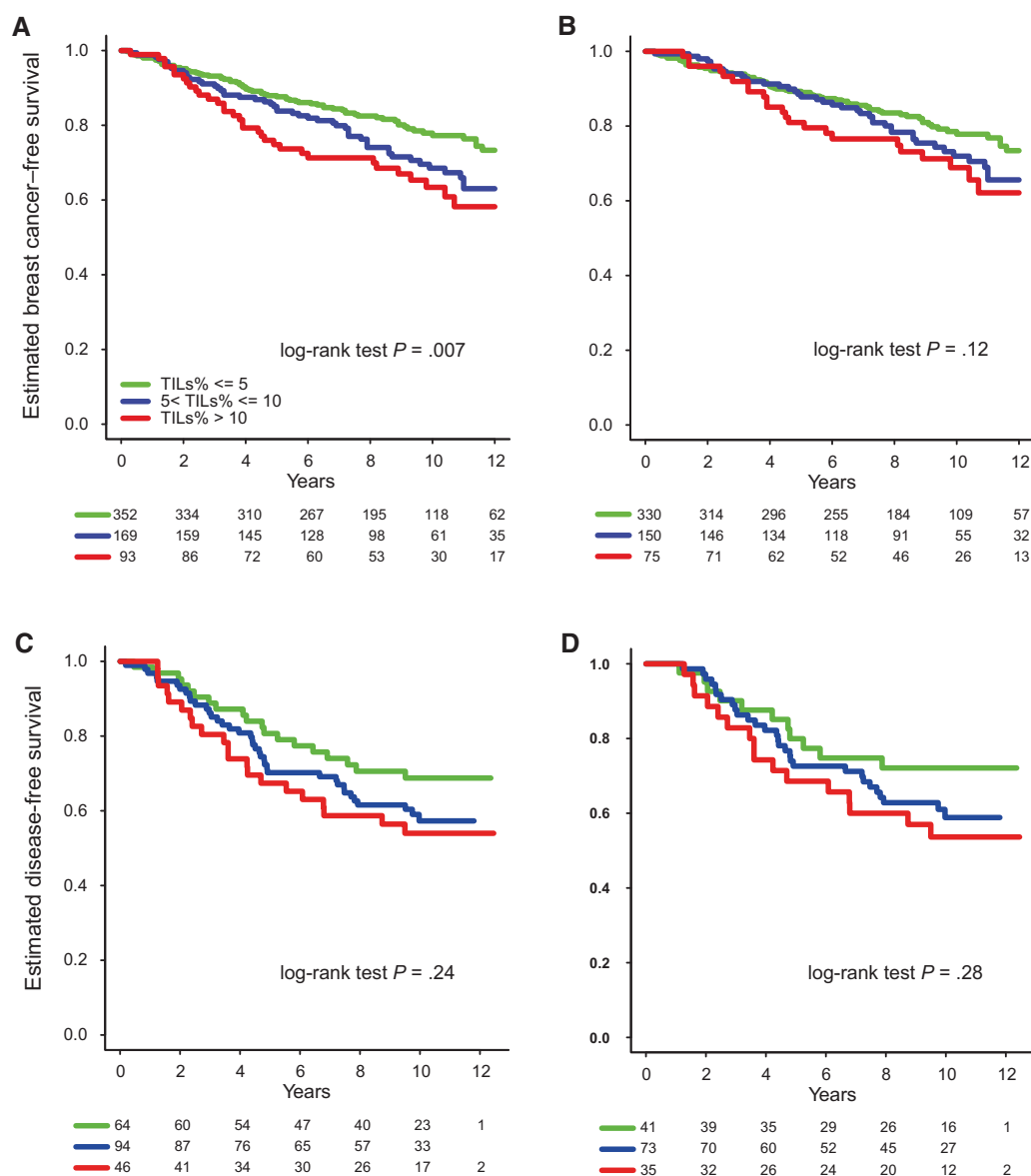


Figure 5. Association between tumor-infiltrating lymphocytes (TILs) and survival. Kaplan-Meier curves displaying the estimated breast cancer-free interval probability according to the TIL group (low: $\leq 5\%$, intermediate: >5 and $\leq 10\%$, high: $>10\%$) in all invasive lobular carcinomas (ILCs) (A) and only the estrogen receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative ILC of the retrospective series (B). Kaplan-Meier curves displaying the estimated disease-free survival probability according to the TIL group in all ILCs (C) and the only the ER-positive/HER2-negative ILC (D) from the BIG 02-98 series. All statistical tests were two-sided. ER = estrogen receptor; ILC = invasive lobular carcinoma; TIL = tumor-infiltrating lymphocyte.

CD3⁺, CD4⁺, CD8⁺, CD68⁺, and FOXP3⁺ cells on the Nottingham series between these two main breast cancer histologies, we noticed that while no difference was observed for total CD8⁺ levels, intratumoral CD8⁺ levels were statistically significantly lower in ER-positive/HER2-negative ILC compared with IDC, with very rare ILC cases presenting with intratumoral CD8⁺ cells, suggesting that ILC might present an immune-excluded phenotype where immune cells are retained in the stroma, therefore limiting cancer immunity. As tumors with intratumoral CD8⁺ cells have been associated with the best outcome (41), ILC tumors with high TIL levels might be associated with a worse prognosis because of the lack of intratumoral CD8⁺ cells. While the initial transcriptomic comparison between the less and highly infiltrated groups showed similarities with previously reported immune signatures, the CIBERSORT analyses

further revealed statistically significant differences in immune composition between infiltrated ER-positive/HER2-negative IDC and ILC tumors. Whether the strong underrepresentation of M1 macrophages or follicular helper T cells in ILC compared with IDC could further explain our prognostic observations needs to be further investigated.

A unique advantage of our retrospective cohort was the ability to investigate the association between genomic alterations and TILs. Tumors presenting *ARID1A*, *BRCA2*, and *TP53* mutations, as well as 8q24.23 loss and 7p21.2 gains, were associated with higher TIL counts. Although we could not find previous evidence for the association of *ARID1A* mutations and TILs in breast cancer, a study of hepatocellular carcinoma demonstrated that hepatocyte-specific deficiency of *ARID1A* leads to the infiltration of innate immune cells (42). Similarly, in

colorectal cancer, *ARID1A* mutations are known to be more frequent in microsatellite-unstable tumors, and these tumors are also associated with higher immune expression scores (43). It has to be noted, however, that *ARID1A* mutations were previously demonstrated to be enriched in ER-positive/HER2-negative ILC (~6%) compared with ER-positive/HER2-negative IDC (<2%) (13). Regarding *BRCA2*, our observation is in line with the study of Bane et al., who reported that *BRCA2*-mutated tumors exhibited lymphocytic infiltration at the periphery of the tumor (44). While *TP53* mutations have been shown to be associated with higher levels of TILs in ovarian cancer (45), lymphocytic infiltration in basal-like breast cancer has only recently been reported to be associated with the retention of wild-type *TP53* (46). While some evidence is now beginning to accumulate pertaining to the clinical utility of mutation burden, the role of TILs and genomic alterations as potential biomarkers for immunotherapy needs to be further investigated.

We recognize that this study has limitations, which are mainly due to its retrospective nature and the clinical and pathological heterogeneity of the cohorts that were analyzed. Nevertheless, this study represents, to the best of our knowledge, the largest study investigating immune infiltration in ILC.

To conclude, we have shown, in a large cohort and using a standardized methodology for TIL assessment, that most ILCs were characterized by generally low but variable levels of lymphocytic infiltration. We further demonstrated that higher TIL levels were associated with worse prognosis in ILC, but only at the univariate level. The association of TILs with outcome in ER-positive/HER2-negative breast cancer patients should be further investigated in additional large trials to understand whether the association is limited to ILC or not, and whether high lymphocytic infiltration could drive endocrine resistance as previously suggested (47). We further highlighted several differences in terms of immune composition and localization between ILC and IDC, suggestive of an immune-exclusion phenotype. Finally, we showed that higher TIL levels were found in association with specific mutations and copy number alterations. Overall, these observations suggest that that immune infiltration could play a different role in ILC compared with IDC. Further research is needed to understand whether and how immunomodulators could reestablish and promote the antitumor immune response in ILC.

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Notes

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