Heterogeneity of Tumour Infiltrating Lymphocytes (TILs) in Breast Cancer and its prognostic significance

Maryam Althobiti[1], Mohammed A Aleskandarany[1], Chitra Joseph[1], Michael Toss[1], Nigel Mongan[2, 3], Maria Diez-Rodriguez[1], Christopher C Nolan[1], Ibraheem Ashankyty[4], Ian O Ellis [1], Andrew R Green[1], Emad A Rakha[1]

[1]Nottingham Breast Cancer Research Centre, Division of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB, UK.

[2]School of Veterinary Medicine and Science, Sutton Bonington Campus, the University of Nottingham, Sutton Bonington, LE12 5RD, UK, [3] Department of Pharmacology, Weill Cornell Medicine, 1300 York Avenue, New York, NY, 10065, USA

[4]Molecular Diagnostics and Personalised Therapeutics Unit, University of Ha'il, Ha'il 2440, Saudi Arabia.

Correspondence:

Professor Emad A Rakha

Department of Histopathology, Nottingham University Hospital NHS Trust,

City Hospital Campus, Hucknall Road, Nottingham, NG5 1PB, UK

Tel: (44) 0115-9691169, Fax: (44) 0115-9627768

Email: emad.rakha@nottingham.ac.uk, Emad.rakha@nuh.nhs.uk

The authors declare no potential conflicts of interest.

ABSTRACT

Background: Tumour-infiltrating lymphocytes (TILs) in breast cancer (BC) confer prognostic and predictive information. This study aims to assess the spatial and temporal heterogeneity of TILs in BC and its relationship with immune cell subtypes.

Method: Immunohistochemically-defined immune cell subtypes; T-cell markers (CD3, CD8, and FOXP3), B-cell marker (CD20) and histiocytic marker (CD68) were evaluated in a large series (n=1,165) of invasive BC. A subset of full-face haematoxylin and eosin (H&E) stained slides were examined for TILs heterogeneity within primary tumours and the corresponding local recurrent carcinomas to report on spatial and temporal TILs heterogeneity. H&E stained sections from multiple tumour blocks (3-4 blocks per case) representing different tumour areas were evaluated to assess TILS inter-slide heterogeneity as well as intra-slide heterogeneity. Both average (AV-TILs) and hotspot (HS-TILs) stromal TILs were assessed.

Results: AV-TILs showed association with all immune cell subtypes however; the main component were CD3+ cells (mean number = 55) whereas CD20+cells comprised the least component (the mean number = 13). There was no significant statistical difference between TILs across tumour blocks of the same case ($p=0.251$ for AV-TILs and $p=0.162$ for HS-TILs). Triple negative breast cancer (TNBC) showed higher TILs compared with other BC subtypes ($p<0.001$). High AV-TILs, CD3+, CD8+, and CD20+ cells were associated with longer survival in TNBC ($p<0.05$). High AV-TILs in recurrent tumours showed significant association with shorter post-recurrence survival ($p=0.004$).

Conclusion: Despite the heterogeneity of immune cell type components, average TILs in one full-face H&E stained section reliably represent whole tumour TILs. TILs were associated with outcome in TNBC as well as provided prognostic significance in recurrent tumour.

Keywords: breast cancer, TILs, outcome, heterogeneity

Running title: TILs heterogeneity in breast cancer

INTRODUCTION

Breast cancer (BC) microenvironment comprises a complex mixture of cell types including immune modulating cells that provide an opportunity for therapeutic modulation [1, 2]. Several studies have reported prognostic and predictive roles of tumour infiltrating lymphocytes (TILs) in BC as assessed on haematoxylin and eosin (H&E) stained sections regardless of their cellular subtypes [3-6] and guideline recommendations to standardise TILs assessment in BC have been published [7]. However, the impact of TILs heterogeneity, with variable TILs density and components in different tumour areas within BC subtypes remains to be defined. Various TILs subtypes have different roles in tumour progression either suppressing tumour growth by destroying cancer cells or promoting tumour progression by selecting those tumour cells that could survive in an immunocompetent host [8-10]. For instance, T-lymphocytes, which account for the majority of TILs in invasive BC, can activate host defense mechanisms [9] mainly through cytotoxic CD8+ T-cells. However, T regulatory cells (T-reg) play a vital role in immune tolerance to tumour cells and can protect against tumour development and progression [10-12]. The contribution of each immune cell subtype to the prognostic value of overall TILs assessed on H&E slides also remains unclear. In addition, the role of histiocytes, as a component of the immune cells in BC, and whether they contribute prognostic value and should be included in TILs assessment in BC has not been resolved.

This study aims to investigate i) the TILs infiltrate in invasive BC and its spatial distribution and relate this to prognostic parameters and patient outcome in primary and recurrent tumours. ii) Contribution of various immune cell subtypes, as molecularly defined, to overall TILs and their prognostic value were also evaluated.

MATERIALS AND METHODS

Study cohort

The study cohort comprised 1,165 invasive BCs derived from the retrospective Nottingham Primary Breast Carcinoma Series of patients presenting to Nottingham City Hospital between 1988 to 1998. Immunohistochemical detection of a panel of T lymphocytes markers including pan T-cell CD3, cytotoxic T-cell CD8, T-reg FOXP3, B-cell CD20, and Histiocytic cell marker; CD68 were previously performed [13-15]. Full-face H&E stained sections from a subset of cases (n=230) were used to assess TILs infiltration as well as intra-slide heterogeneity. Moreover, to assess inter-slide TILs heterogeneity, H&E stained slides from multiple formalin fixed paraffin embedded (FFPE) archival tissue blocks (3-4 blocks/case) were prepared from a sub-group of cases (n=52/230). Patients' clinical and pathological data including age at presentation, histological tumour type, primary tumour size, axillary lymph node status, histological tumour type, Nottingham Prognostic Index (NPI), Lymphovascular Invasion (LVI) and adjuvant chemotherapy were available. Biological data on expression of ER, PgR, HER2, EGFR, androgen receptor (AR) and basal cytokeratins (CK5/6 and CK14) was also available [16, 17]. The data on HER2 was already available in this early-stage breast cancer cohort and details of staining and scoring were included in previous publications[20-23]. Survival data was prospectively maintained including 1) breast cancer-specific survival (BCSS), defined as the time (in months) from the date of primary surgical treatment to the time of death from BC and 2) distant metastasis free survival (DMFS), defined as the time from the surgery until the first event of distant metastasis. In this study, post recurrence survival was defined as the time (in months) from the date of surgical removal of the recurrent tumour to the date of death from BC. Of the recurrent cases, 44 patients had a local recurrence tumour tissue avail-

able and these were used to compare TILs distribution between primary and corresponding recurrent tumour.

This study was approved by the Nottingham Research Ethics Committee 2 under the title "Development of a molecular genetic classification of breast cancer".

Immunohistochemistry and Immunoscoring

The total number of each immune cell type and histiocyte were counted in the stroma surrounding the tumour in each core. Supplementary table 1 shows the details of the different antibodies which were used.

TILs assessment on H&E stained sections

All tumour specimens were routinely fixed in 10% formalin and embedded in paraffin. Full-face, 4μm sections were prepared for H&E staining and evaluated for stromal TILs. All cases contained adequate tumour burden with at least 40% of the section surface area in each slide. H&E slides were scanned into high-resolution digital images at 20x magnification using 3D Histech Panoramic 250 Flash II scanner (3DHISTECH Ltd., Budapest, Hungary). The digital images were viewed using the 3D Histech Panoramic Viewer (3DHISTECH Ltd., Budapest, Hungary). TILs were semi-quantitatively assessed following the recommended method of the 2014 International TILs Working Group [7]. Each section was first scanned using low magnification and the regions of interest located within the invasive tumour borders in the stromal compartment; around and dispersed in the intervening stroma between carcinoma cells but not directly contacting tumour cells. TILs around crush artefacts, tumour necrosis, extensive central regressive hyalinisation, or DCIS/normal lobules were not considered for quantification. All mononuclear cells, lymphocytes, histiocytic cells, and plasma cells, if any, were

scored and recorded as a percentage of TILs. The quantity of TILs was evaluated by the percentage of TILs occupied in the stroma (average TILs; AV-TILs). For further quantification of the pattern of TILs infiltrate, the presence of areas with dense TILs infiltrate were further determined and designated as 'Hotspots-TILs' (HS-TILs). HS-TILs was defined as a cluster of lymphocytes densely containing the highest number of lymphocytes which is typically more than that of the overall/average stromal TILs. HS-TILs was equal to AV-TILs only in the cases showing no or occasional scattered lymphocytes or in rare cases showing dense lymphocytic infiltration throughout the tumour. The highest area of TILs density was considered as the HS and was introduced to study intra-slide as well as inter-slides TILs heterogeneity. For assessment of intra-slide heterogeneity, annotation of each digital image was performed by dividing the tumour area into four quadrants using a vertical and horizontal line. AV-TILs and HS-TILs were assessed in each quadrant separately in addition to the whole slide. All cases were scored independently by two observers (M. Althobiti and C. Joseph), blinded to histopathological data and patients' outcome. The observers were trained by a consultant pathologist (ER). Further, to test for the intra-observer reproducibility of our scoring system, 50% of the cases were randomly selected and rescored by the same scorer.

STATISTICAL ANALYSIS

Intra-observer agreement was determined using Pearson correlation coefficient whereas inter-observer agreement was determined using intra-class correlation coefficient. TILs scores were categorised using a 10% cut-off with scores <10% defined as negative/low whereas cases with scores ≥10% were defined as positive/high [18]. In recurrent tumours, the median percentage of TILs scores (5%) was used. Positive immunoreactivity for CD3, CD8, CD20, FOXP3, and CD68 were defined as previously described [13-15, 19]. The association between the categorical groups of TILs density and clinico-pathological parameters were analysed using

Chi-square test. Spearman rank correlation co-efficient was used to assess the relationship between TILs and immune cell subtypes. Friedman and Kendall's test was used for testing TILs expression between four slides (inter-slide heterogeneity) and in four quadrants (intra-slide heterogeneity). Association with patient outcome was assessed using Kaplan-Meier and log-rank test. Cox proportional hazards regression models were built for multivariate survival analyses to estimate the hazard ratio (HR) of TILs and immune cell subtypes adjusted by other established prognostic factors. A p-value of less than 0.05 (two- tailed) was considered significant in all statistical tests.

RESULTS

Immune cell subtypes and TILs

In the study cohort, 74.7% of the tumours were ER positive, 59% were PR+ positive and 9.3% were HER2 positive. To assess specific sub-population of TILs, the expression of lymphocytic biomarkers; CD3, CD8, CD20, and FOXP3 and macrophage/histiocytic biomarker; CD68 were evaluated in the whole cohort (n=1,165). Supplementary Table 2 shows the distribution of immune subtypes within different molecular classes. This revealed that the T-lymphocyte population, as demonstrated by pan-T (CD3+ cells), was the main component of TILs followed by macrophages (CD68+ cells), while B-lymphocytes (CD20+ cells), comprised the least common component. Among T-lymphocytes, the overall contribution of CD8+ cells was more frequent than FOXP3+ cells. Despite the increased number of all immune cells components in TNBC, the ratio of each subtype appeared similar across the whole series with the CD3+ cell population being the main contributing component (Figure 1).

Univariate survival analysis demonstrated that high FOXP3+ and CD68+ counts were associated with shorter BCSS in all BC subtypes (p=0.006 and p<0.001, respectively; Supplementary Figures 1). However, high CD20+ cells showed a trend toward longer BCSS (p=0.066)

High CD3+ (p=0.021, p=0.011), CD8+ (p=0.017, p=0.012), and CD20+ cells infiltrate (p=0.037, p=0.045) were associated with longer BCSS and DMFS respectively in TNBC (Supplementary Figures 2A-C). Dense histiocytic CD68+ cells were associated with shorter BCSS in luminal B tumours (p=0.04). High CD20+ infiltrate was associated with better BCSS in HER2 positive tumours (p=0.04) (Supplementary Figures 3A&B respectively). Cox-proportional models including patient age, tumour grade, nodal stage and different immune cell subtypes showed only high CD8+ (p=0.002, HR 0.5, 95%CI 0.3– 0.8) and high CD3+ (p=0.003, HR 0.3, 95%CI 0.2– 0.7) lymphocytic infiltrates were independent predic-

tors of improved outcome in TNBC while CD20+, CD68+ and FOXP3+ cells were not significantly associated with outcome (supplementary table 3).

TILs morphological heterogeneity

We observed high reproducibility of TILs scoring by the same observer (Pearson correlation coefficient: 0.93 for AV-TILs and 0.97 for HS-TILs), and between the two observers (intra-class correlation coefficient: 0.79 for AV-TILs and 0.83 for HS-TILs) and (kappa agreement: 0.825 for AV-TILs and 0.785 for HS-TILs) using the methodology adopted in this study [7]. Inter-observer agreement was determined using intra-class correlation coefficient for the four slides between the two scorers (0.771 for slide 1, 0.580 for slide 2, 0.682 for slide 3 and 0.910 for slide 4)

The mean, median and range of TILs percent of AV and HS scores in both the primary and recurrent tumours are displayed in supplementary table 4 A&B.

No significant differences between slides representing various tumour blocks/areas were identified (inter-slide variation, p=0.251 for AV-TILs and p=0.162 for HS-TILs) (supplementary Figure 4). Moreover, our result showed that patients with the luminal A tumours were more homogenous in respect of inter-slide AV-TILs expression than other subtypes. When each slide was divided into four quadrants and each quadrant scored independently, the variation of TILs was statistically significant (intra-slide variation, p=0.019 for AV-TILs and p<0.0001 for HS-TILs). Interestingly, recurrent tumours showed a trend towards lower TILs scores (p=0.052) and showed homogeneous TILs distribution when compared with their corresponding primary tumour (Figure 2). AV-TILs was higher in TNBC compared with other BC classes (p<0.001). Significant multiple correlations between AV-TILs and different im-

mune cell subpopulations and between the immune cells markers were identified (all $p<0.001$).

TILs (AV and HS) in primary BC were positively associated with higher tumour grade ($p<0.001$), tumour stages ($p=0.038$), poor NPI prognostic group ($p<0.001$), TNBC ($p<0.001$), and AR negative phenotypes ($p=0.012$). No significant association between TILs and patients age, tumour size, lymph node stage or LVI status was identified (Table 1). TILs (AV and HS) showed a positive association with the grade in TNBC. Interestingly, within TNBC, AR positivity was associated with lower scores of TILs ($p<0.001$). No association was identified between TILs scores and basal-like phenotype as defined by basal cytokeratin and EGFR [17]. Univariate survival analysis showed no significant association between TILs (AV and HS) and patient BCSS either in the whole cohort or those with local recurrence.

High AV-TILs showed significant association with longer BCSS in TNBC patients ($p=0.026$), where distant metastasis developed in only 91 cases out of 230 cases. However, 61 cases of TNBC showed TILs associated with longer distant metastasis free survival ($p=0.017$) (Figure 3). This was not observed in other BC subtype. Notably, survival analysis between treated and untreated patients (chemotherapy and endocrine therapy) did not show a significant association. However, in TNBC AV-TILs was a prognostic factor independent of adjuvant chemotherapy treatment. No significant association between HS-TILs and BCSS or DMFS was identified in any of BC subtypes.

As AV-TILs was a significant predictor of outcome in TNBC, further analyses were limited to TNBC. Cox-proportional models including other prognostic parameters including patient age, tumour grade, and nodal stage showed that AV-TILs was an independent predictor for good

prognosis [p=0.004, HR 0.4, 95% CI 0.2– 0.8). In the model including TILs and various immune cells (CD3, CD8, FOXP3, CD20 and CD68), only AV-TILs showed significant association with patient outcome (supplementary Table 5).

Testing the prognostic power of the combination of positive/negative TILs with high/low immune biomarker was performed, however there was no significant association in all breast cancer subtypes. Interestingly, in recurrent tumours, there was a significant association between high AV-TILs density and shorter post-recurrence patient survival (p=0.004) (Figure 4).

DISCUSSION

There is a growing interest in quantifying and reporting total TILs in BC and their different subpopulations in clinical practice due to their prognostic and possibly predictive value for immunotherapies [24, 25]. In this study, a large cohort with long-term clinical follow-up was used to assess different immune cells infiltrate and their expression pattern in BC as well as correlating with patient outcome and tumour progression.

The International TILs Working Group has reported that in most tumours, there is no obvious inter-slide heterogeneity (i.e. between multiple tissue sections from different tumour blocks of the same case) in TILs content at the morphological level. However, no robust evidence to support this observational statement has yet been provided [7]. To our knowledge, ours is the first study to assess TILs heterogeneity utilising multiple tumour tissue blocks from the same primary tumour and comparing these with their corresponding recurrence. For each sample in this study, TILs were assessed as average (AV-TILs) and hotspots (HS-TILs) to elaborate upon TILs distribution in different tumour areas. Our results showed no significant differences in the overall TILs scores between tumour blocks whether assessed as AV-TILs or HS-TILs, indicating that any full-face H&E breast tumour tissue section is sufficient to evaluate TILs density within a whole tumour. However, when the analysis was limited to smaller tumour areas as per different areas of the same slide (intra-slide heterogeneity), the difference in TILs scores were significant, particularly with regard to hotspots.

The latter finding represents the presence of substantial intra-slide heterogeneity and therefore underscores the variable nature of immune infiltrate within different tumour cells population. This is consistent with earlier reports, where CD8, CD3, and CD20 expressing TILs subpopulations in core biopsies were more heterogeneous across the core compared with multiple cores from the same tumour [26]. The variation of CD3, CD8 and CD20; were 30

-33% between the cores biopsy from same tumour while the higher variation 66-69% was in the same section[27]. Another study reported that spatial heterogeneity of TILs subtypes could play role in patient's outcome. For instance, high CD3+ T lymphocytes in invasive margin is associated with disease free survival in BC, where high total and distance CD8+ T lymphocyte corresponded with better outcome in BC[28]. Both assessment methods of TILs; AV-TILs and HS-TILs, were significantly associated with other prognostic parameters in the whole cohort [28].

Interestingly, TILs showed lower proportions and were mostly homogenously distributed in recurrent tumours compared with their corresponding primary tumours. This is consistent with a previous report where lower expression of TILs in metastatic recurrences of TNBC and HER2 positive BC were observed compared to primary tumours [29]. Moreover, significantly shorter post-recurrence survival was observed in recurrent patients with dense TILs, providing evidence that assessment of TILs in recurrent tumours can be used as a prognostic variable to predict post-recurrence outcome. Of note, the recurrent tumours included all BC subtypes. Thus, the inverse association could be explained, at least in part, by the heterogeneity of BC and the variation of TILs interaction with primary and recurrent tumour as well the different subtypes.

This study showed that TILs sub-populations were differently expressed in BC tumours where T-cells, as defined by CD3+ cells, appeared to dominate. This indicates that TILs are a heterogeneous mixture of different immune cell types that are most likely recruited to the tumour vicinity to perform specific functions [30, 31]. Additionally, our data showed that CD3, CD8, FOXP3, CD20 and CD68 were significantly associated with overall TILs, and TILs were higher in the more aggressive TNBC subtype than others. It has been reported that TILs assessed in H&E sections are significantly associated with CD20+ and CD45+ in BC[32]. The

high CD8+ and CD3+ cellular infiltrate showed a good prognosis and independent predictive value in TNBC regardless of other clinicopathological parameters. These findings are concordant with the results reported by other studies [15, 33, 34]. The higher CD20+ lymphocytes within the tumour were associated with better outcome in TNBC and HER2+. This may point to the higher immunogenicity of TNBC and HER2+ BC than luminal BC due to producing more B-cell antibodies against the tumour cell; results consistent with those reported by Buisseret and colleagues [35]. Several subtypes of TNBC with their clinical relevance have been described with better outcome or treatment responses[36]. However, there was no significant association between FOXP3+ and patient outcome in TNBC. In contrast to our observations, Yeong et al reported that high density of intra tumoral FOXP3+ was associated with longer overall survival but not with stromal infiltration[37] . In our study, lower TILs scores were observed in the AR positive subclass of TNBC which is considered as prime candidates for anti-androgen and CDK4/6 inhibition therapies [38, 39]. However, no differential expression of TILs or various immune cell subtypes was observed with regard to sub-classification of TNBC into basal-like and non-basal-like groups [17].

High expression of CD68 was significantly associated with worse outcome in patients with luminal B tumours, which may explain, at least in part, the distinct roles of TILs sub-population in different BC subtypes [40]. These results also underscore a distinct tumour stromal interactions in TNBC compared to non-TNBC tumours as evidenced by their different inflammatory microenvironment. The tumour association macrophage differentiates to active classic (M1) and alternative (M2) where the latter is associated with growth, angiogenesis, migration and invasion of a diversity of cancers[41].

The significant association of AV-TILs with different lymphocyte subtypes and its independent association with outcome in this study signify the reliability of AV-TILs as assessed on

H&E stained sections for accurate immune cell scoring as a prognostic parameter in BC. CD3 and CD8 lost their prognostic effect when they were included in the same model. This provides evidence for the utility of prognostic value of AV-TILs assessment in H&E stained sections, particularly in TNBC.

In conclusion, TILs infiltrate is significantly different between TNBC and non-TNBC with distinct subpopulations of immune cells in the former. This might explain the different prognostic utility of TILs infiltrate and the immune cells subtypes in BC. Although the International TILs International Working Group have recognised heterogeneity as an issue, no data is available to provide any strong guidance. Our result confirms that average TILs assessment using one representative tumour block can reliably report on TILs infiltrate across a whole breast tumour. Assessing average TILs score using H&E slides not only provides reproducible and independent prognostic information in TNBC but is also equivalent to immunohistochemically defined subpopulations of immune cells. The role of B-lymphocytes and histiocytes in BC biology and prognosis as well as the association between TILs and the outcome in the recurrent tumour, warrant further investigation.

Legends of the figures

Figure 1 Representative photomicrographs of the expression of immune cell markers (CD3, CD8, FOXP3, CD20, and CD68)

Figure 2 Representative photomicrographs of the difference between tumour proportions of TILs infiltrate in the same case for primary (A, B) and its corresponding recurrent tumour (C, D)

Figure 3: Kaplan Meier survival plots for average TILs % expression in triple negative breast cancer for A) breast cancer specific survival (BCSS) & B) Distant Metastasis Free Survival (DMFS)

Figure 4: Kaplan Meier survival plots for TILs % expression in recurrent breast cancer showing the post-recurrence survival

References

1. Place AE, Jin Huh S, Polyak K. The microenvironment in breast cancer progression: Biology and implications for treatment. *Breast cancer research : BCR* 2011;**13**;227.
2. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nature reviews. Cancer* 2009;**9**;239-252.
3. Tian T, Ruan M, Yang W, Shui R. Evaluation of the prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers. *Oncotarget* 2016;**7**;44395-44405.
4. Hida AI, Sagara Y, Yotsumoto D *et al.* Prognostic and predictive impacts of tumor-infiltrating lymphocytes differ between triple-negative and her2-positive breast cancers treated with standard systemic therapies. *Breast Cancer Res Treat* 2016;**158**;1-9.
5. 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**;1544-1550.
6. Adams S, Gray RJ, Demaria S *et al.* Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase iii randomized adjuvant breast cancer trials: Ecog 2197 and ecog 1199. *J Clin Oncol* 2014;**32**;2959-2966.
7. Salgado R, Denkert C, Demaria S *et al.* The evaluation of tumor-infiltrating lymphocytes (tils) in breast cancer: Recommendations by an international tils working group 2014. *Ann Oncol* 2015;**26**;259-271.
8. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nature medicine* 2013;**19**;1423-1437.
9. Wakabayashi O, Yamazaki K, Oizumi S *et al.* Cd4+ t cells in cancer stroma, not cd8+ t cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers. *Cancer Sci* 2003;**94**;1003-1009.
10. Liu S, Lachapelle J, Leung S, Gao D, Foulkes WD, Nielsen TO. Cd8+ lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. *Breast cancer research : BCR* 2012;**14**;R48.
11. Bailey SR, Nelson MH, Himes RA, Li Z, Mehrotra S, Paulos CM. Th17 cells in cancer: The ultimate identity crisis. *Frontiers in immunology* 2014;**5**;276.
12. DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: Crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast cancer research : BCR* 2007;**9**;212-212.
13. Mahmoud SM, Paish EC, Powe DG *et al.* Tumor-infiltrating cd8+ lymphocytes predict clinical outcome in breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011;**29**;1949-1955.
14. Mahmoud SM, Paish EC, Powe DG *et al.* An evaluation of the clinical significance of foxp3+ infiltrating cells in human breast cancer. *Breast cancer research and treatment* 2011;**127**;99-108.
15. Mahmoud SM, Lee AH, Paish EC, Macmillan RD, Ellis IO, Green AR. The prognostic significance of b lymphocytes in invasive carcinoma of the breast. *Breast Cancer Res Treat* 2012;**132**;545-553.
16. Aleskandarany MA, Abduljabbar R, Ashankyty I *et al.* Prognostic significance of androgen receptor expression in invasive breast cancer: Transcriptomic and protein expression analysis. *Breast Cancer Res Treat* 2016;**159**;215-227.
17. Rakha EA, Elsheikh SE, Aleskandarany MA *et al.* Triple-negative breast cancer: Distinguishing between basal and nonbasal subtypes. *Clin Cancer Res* 2009;**15**;2302-2310.
18. Hida AI, Sagara Y, Yotsumoto D *et al.* Prognostic and predictive impacts of tumor-infiltrating lymphocytes differ between triple-negative and her2-positive breast cancers treated with standard systemic therapies. *Breast Cancer Research and Treatment* 2016;**158**;1-9.
19. Mahmoud SM, Lee AH, Paish EC, Macmillan RD, Ellis IO, Green AR. Tumour-infiltrating macrophages and clinical outcome in breast cancer. *Journal of clinical pathology* 2012;**65**;159-163.

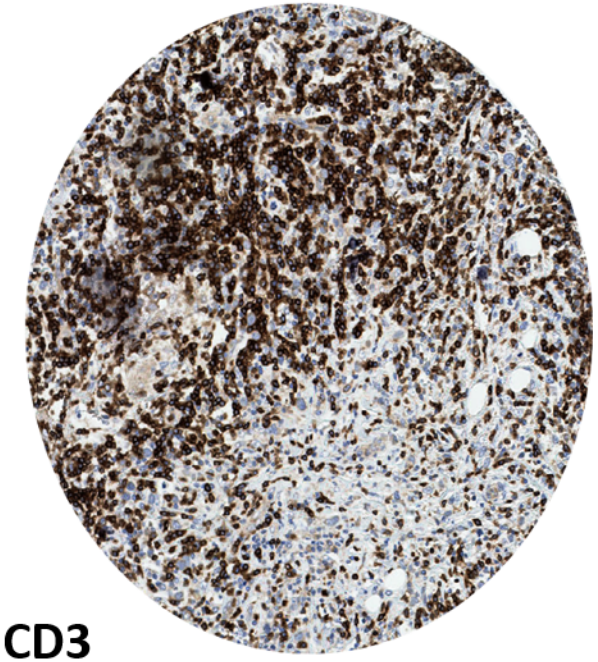
20. Garcia-Caballero T, Grabau D, Green AR *et al.* Determination of her2 amplification in primary breast cancer using dual-colour chromogenic in situ hybridization is comparable to fluorescence in situ hybridization: A european multicentre study involving 168 specimens. *Histopathology* 2010;**56**;472-480.
21. El Ansari R, Craze ML, Miligy I *et al.* The amino acid transporter slc7a5 confers a poor prognosis in the highly proliferative breast cancer subtypes and is a key therapeutic target in luminal b tumours. *Breast cancer research : BCR* 2018;**20**;21.
22. Green AR, Aleskandarany MA, Agarwal D *et al.* Myc functions are specific in biological subtypes of breast cancer and confers resistance to endocrine therapy in luminal tumours. *British journal of cancer* 2016;**114**;917-928.
23. Abd El-Rehim DM, Ball G, Pinder SE *et al.* High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cdna expression analyses. *International journal of cancer* 2005;**116**;340-350.
24. Stanton SE, Disis ML. Clinical significance of tumor-infiltrating lymphocytes in breast cancer. *J Immunother Cancer* 2016;**4**;59.
25. Wang K, Xu J, Zhang T, Xue D. Tumor-infiltrating lymphocytes in breast cancer predict the response to chemotherapy and survival outcome: A meta-analysis. *Oncotarget* 2016;**7**;44288-44298.
26. Brown JR, Wimberly H, Lannin DR, Nixon C, Rimm DL, Bossuyt V. Multiplexed quantitative analysis of cd3, cd8, and cd20 predicts response to neoadjuvant chemotherapy in breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014;**20**;5995-6005.
27. Mani NL, Schalper KA, Hatzis C *et al.* Quantitative assessment of the spatial heterogeneity of tumor-infiltrating lymphocytes in breast cancer. *Breast cancer research : BCR* 2016;**18**;78.
28. Nawaz S, Heindl A, Koelble K, Yuan Y. Beyond immune density: Critical role of spatial heterogeneity in estrogen receptor-negative breast cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2015;**28**;1621.
29. Ogiya R, Niikura N, Kumaki N *et al.* Comparison of tumor–infiltrating lymphocytes between primary and metastatic tumors in breast cancer patients. *Cancer science* 2016;**107**;1730-1735.
30. Oelkrug C, Ramage JM. Enhancement of t cell recruitment and infiltration into tumours. *Clinical and experimental immunology* 2014;**178**;1-8.
31. Zhang Y, Gallastegui N, Rosenblatt JD. Regulatory b cells in anti-tumor immunity. *International immunology* 2015;**27**;521-530.
32. Buisseret L, Desmedt C, Garaud S *et al.* Reliability of tumor-infiltrating lymphocyte and tertiary lymphoid structure assessment in human breast cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2017;**30**;1204-1212.
33. Sikandar B, Qureshi MA, Naseem S, Khan S, Mirza T. Increased tumour infiltration of cd4+ and cd8+ t-lymphocytes in patients with triple negative breast cancer suggests susceptibility to immune therapy. *Asian Pac J Cancer Prev* 2017;**18**;1827-1832.
34. Denkert C, Loibl S, Noske A *et al.* Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2010;**28**;105-113.
35. García-Teijido P, Cabal ML, Fernández IP, Pérez YF. Tumor-infiltrating lymphocytes in triple negative breast cancer: The future of immune targeting. *Clinical Medicine Insights. Oncology* 2016;**10**;31-39.
36. Burstein MD, Tsimelzon A, Poage GM *et al.* Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2015;**21**;1688-1698.
37. Yeong J, Thike AA, Lim JC *et al.* Higher densities of foxp3(+) regulatory t cells are associated with better prognosis in triple-negative breast cancer. *Breast Cancer Res Treat* 2017;**163**;21-35.

38. Barton VN, Gordon MA, Richer JK, Elias A. Anti-androgen therapy in triple-negative breast cancer. *Therapeutic Advances in Medical Oncology* 2016;**8**;305-308.
39. Mina A, Yoder R, Sharma P. Targeting the androgen receptor in triple-negative breast cancer: Current perspectives. *OncoTargets and therapy* 2017;**10**;4675-4685.
40. Medrek C, Ponten F, Jirstrom K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC cancer* 2012;**12**;306.
41. Yang J, Li X, Liu X, Liu Y. The role of tumor-associated macrophages in breast carcinoma invasion and metastasis. *International Journal of Clinical and Experimental Pathology* 2015;**8**;6656-6664.

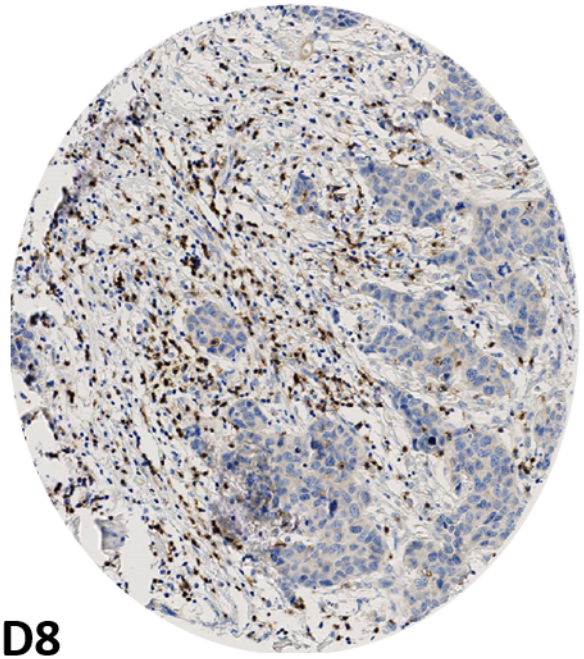
Table 1: Associations of tumour infiltrating lymphocytes (Average TILs) and clinicopathological parameters in breast cancer (n=230)

Parameters	**Whole cohort (n=1165)**	**TILs H&E subgroup (n=230)**		
	N (%)	**Low TILs No. (%)**	**High TILs No. (%)**	$\chi 2$ **p-value**
Patient Age (Years) < 50 ≥ 50	 818(66.0) 421(34.0)	 44 (37.9) 31 (27.4)	 72 (62.1) 82 (72.6)	2.9 0.091
Tumour size (cm) < 2 ≥ 2	 600(48.8) 630(51.2)	 44 (28.6) 31 (41.3)	 110 (71.4) 44 (58.7)	3.7 **0.038**
Lympho-vascular Invasion Negative Positive	 855(69.7) 855(69.7)	 52 (34.9) 23 (28.8)	 97(65.1) 57(71.3)	0.9 0.344
Tumour Grade Grade I Grade II Grade III	 257(19.6) 443(33.7) 612(46.6)	 12 (75.0) 19 (55.9) 43 (24.2)	 4 (25.0) 15 (44.1) 135 (75.8)	27.3 **<0.001**
Axillary nodal stage * Stage I Stage II Stage III	 897(66.5) 357(26.5) 94(7.0)	 53 (37.9) 17 (28.3) 4 (14.3)	 87 (62.1) 43 (71.7) 24 (85.7)	6.5 **0.038**
Nottingham Prognostic Index Good Prognostic Group Moderate Prognostic Group Poor Prognostic Group	 187(15.2) 629(51.1) 414(33.7)	 9 (15.8) 49 (33.3) 17 (68.0)	 48 (84.2) 98 (66.7) 8 (32.0)	21.6 **<0.001**
Oestrogen Receptor Negative Positive	 329(25.3) 973(74.7)	 36 (21.8) 39 (60.9)	 129 (78.2) 25 (39.1)	32.1 **<0.001**
Progesterone Receptor Negative Positive	 483(40.6) 708(59.4)	 43 (24.2) 32 (62.7)	 135 (75.8) 19 (37.3)	26.8 **<0.001**
HER2 status Negative Positive	 1063(73.2) 135(9.3)	69 (32.5) 5 (31.3)	 143 (67.5) 11 (68.8)	0.1 0.915
Triple negative status Non-triple negative Triple negative	 1052(83.2) 213(16.8)	41 (55.4) 33 (21.44)	 33 (44.6) 121 (78.6)	26.3 **<0.001**

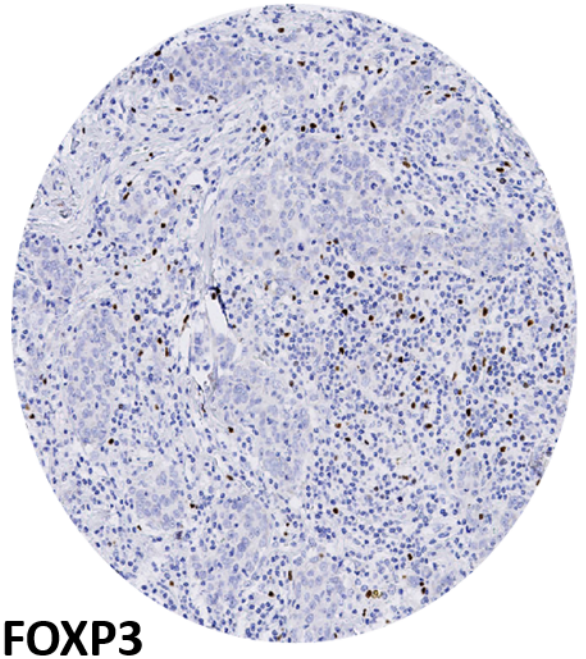
*** Nodal status based on Nottingham system nodal stage (1 for node negative, 2 = 1-3 nodes positive, and 3 ≥ 4 nodes positive).**



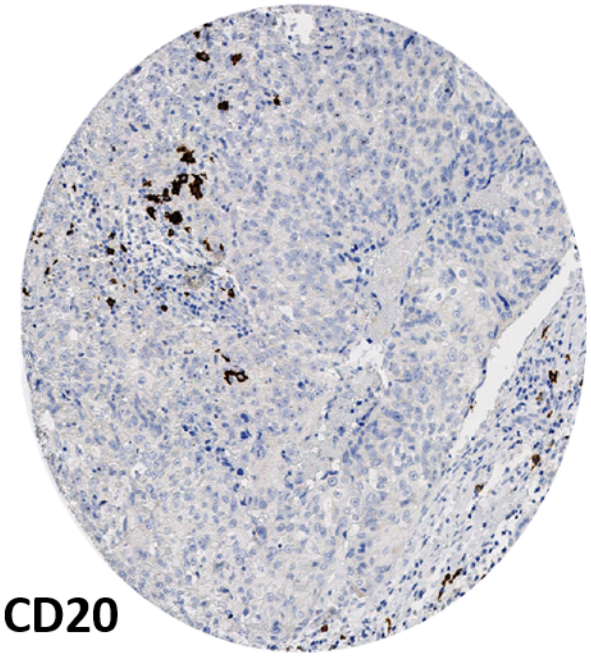
CD3



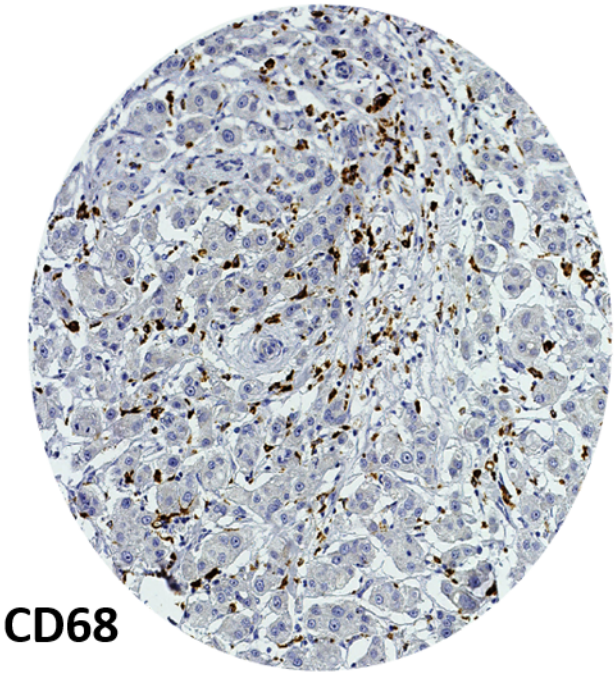
CD8



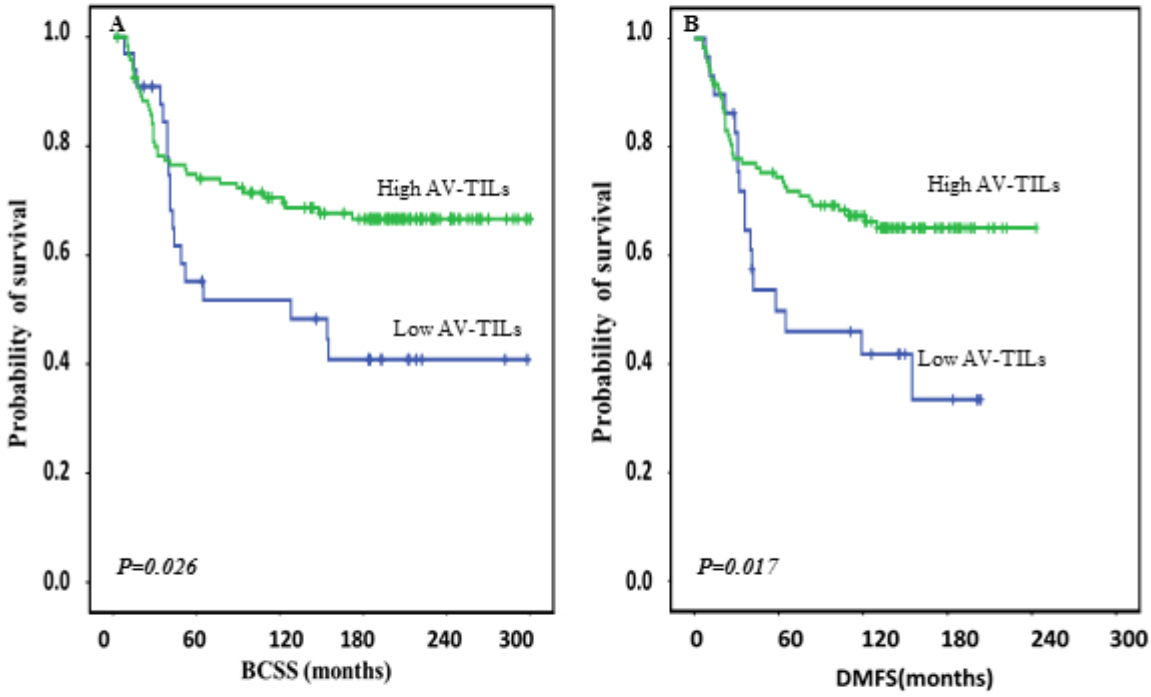
FOXP3



CD20



CD68


A
1.0
0.8
0.6
0.4
0.2
0.0
Probability of survival
High AV-TILs
Low AV-TILs
P=0.026
0
60
120
180
240
300
BCSS (months)
B
1.0
0.8
0.6
0.4
0.2
0.0
Probability of survival
High AV-TILs
Low AV-TILs
P=0.017
0
60
120
180
240
300
DMFS(months)

Supplementary Table 1: Antibodies used in immunohistochemistry.

Antibody	Clone	Dilution	Antigen retrieval	Source
CD3	SP7	1:100	Microwave in EDTA	Vector laboratories
CD8	1A5	1:50	Microwave in citrate	Vector laboratories
FOXP3	23A/E7	1:100	Microwave in EDTA	Abcam
CD20	L26	1:300	Microwave in citrate	Dako
CD68	KP1	1:300	Microwave in citrate	Dako

Supplementary Table 2: Mean, median and range, of immune cell subtypes counts/core diameter assessed as absolute number of cells (in the whole series and in different BC molecular subtypes).

Stromal immune cell density/ core diameter	Whole series			Luminal A			Luminal B			HER2 enriched			Triple negative		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
CD3+	55	33	0-450	34	22	0-258	44	25	0-317	73	51	0-425	93	67	0-450
CD8+	25	11	0-242	16	8	0-142	22	10	0-317	31	17	0-173	40	23	0-242
FOXP3+	14	7	0-153	6	2	0-55	11	5	0-68	19	13	0-153	27	21	1-128
CD20+	13	1	0-415	7	0	0-211	11	1	0-253	22	3	0-415	24	5	0-255

CD68+	32	24	0-252	18	15	0-150	28	23	1-215	38	32	3-252	51	42	1-225

Supplementary Table 3: Multivariate Cox regression hazard model including other prognostic clinicopathological parameters shows that average TILs as well as immunohistochemically defined CD3+ or CD8+ T-lymphocytes provided an independent prognostic value; associated with longer breast cancer specific survival in the subset of triple negative breast cancer.

Variable	**Hazard ratio**	**95% Confidence Interval (CI)**		***p- value***
		Lower	**Upper**	
Patient Age	0.7	0.6	4.2	*0.169*
Tumour Grade	2.9	1.1	3.1	*0.134*
Node Stage	2.0	0.5	2.2	*<0.001*
Tumour Size	1.3	0.7	2.3	*0.291*
TILs-AV	0.4	0.2	0.7	*0.004*
Patient Age	0.6	0.4	1.0	*0.055*
Tumour Grade	1.9	0.6	5.8	*0.252*
Node Stage	1.7	1.2	2.2	*<0.001*
Tumour Size	1.5	0.9	2.4	*0.081*
CD8+	0.4	0.2	0.7	*0.002*
Patient Age	0.6	0.4	1.0	*0.08*
Tumour Grade	1.4	0.7	3.2	*0.998*

Node Stage	1.6	1.2	2.1	*0.001*
Tumour Size	1.4	0.9	2.3	*0.097*
CD3+	0.3	0.1	0.6	*0.003*

Supplementary table 4A: Mean, median and range of %TILs in primary and their corresponding recurrence in subset of study cohort (n=44)

% of stromal TILS/tumour	**Primary tumour**			**Recurrent tumour**		
	Mean	**Median**	**Range**	**Mean**	**Median**	**Range**
AV-TILs	22	15	1-80	9	5	1-45
HS-TILs	38	28	1-90	13	7	1-60

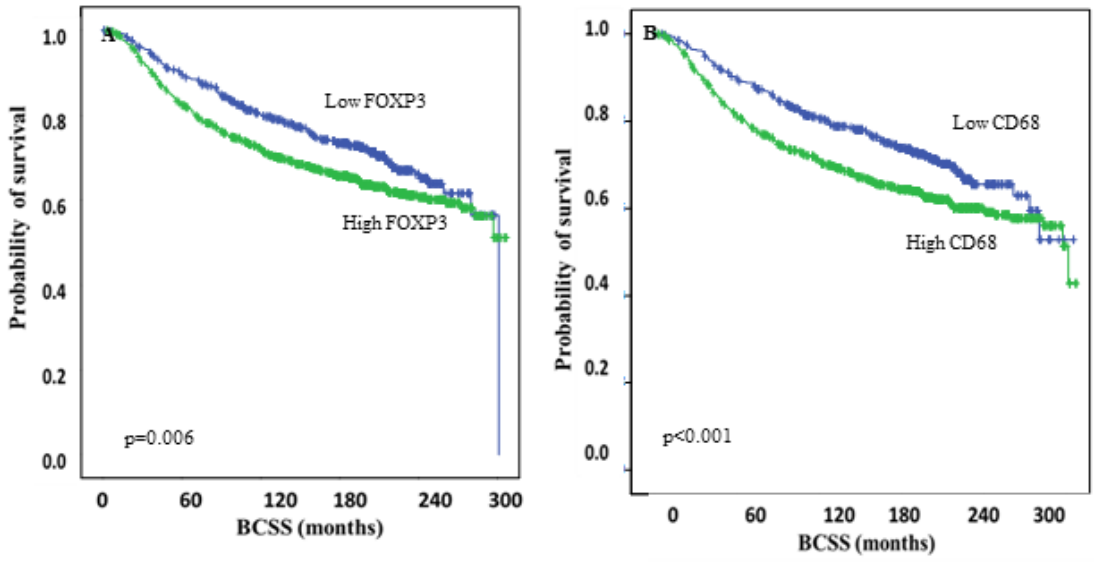
% of stromal TILS/tumour	Luminal A			Luminal B			HER2 enriched			Triple negative		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
AV-TILs												
AV-TILs	8	5	1-40	12	10	3-40	16	12	5-50	27	25	1-85
HS-TILs	16	8	2-90	22	12	3-90	33	20	6-90	45	40	1-90

Supplementary table 4B: Mean, median and range of %TILs in different breast cancer molecular subtype

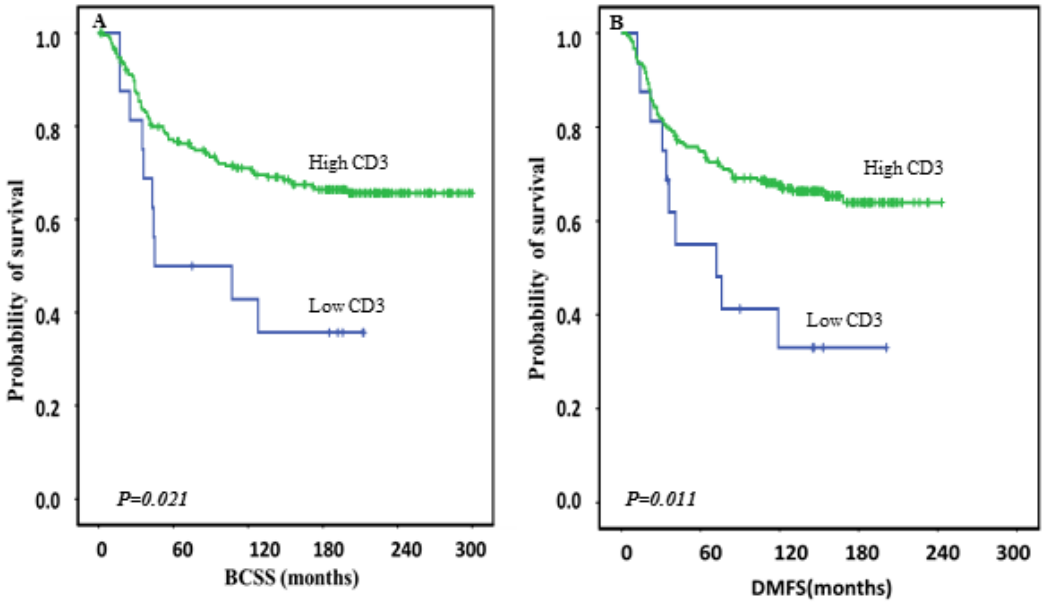
Supplementary table 5: Multivariate Cox regression hazard model including average TILs and various immunohistochemically defined immune cell subtypes shows that average TILs is the only independent prognostic parameter associated with longer breast cancer specific survival in the subset of triple negative breast cancer

Variable	**Hazard ratio**	**95%CI**		***p-value***
		Lower	**Upper**	
CD3+	0.6	0.1	2.8	*0.487*
CD8+	0.5	0.2	1.1	*0.088*
FOXP3+	1.8	0.5	6.1	*0.313*
CD20+	1.1	0.5	2.5	*0.724*
CD68+	2.3	0.7	7.5	*0.140*
AV-TILs	0.3	0.1	0.8	*0.023*

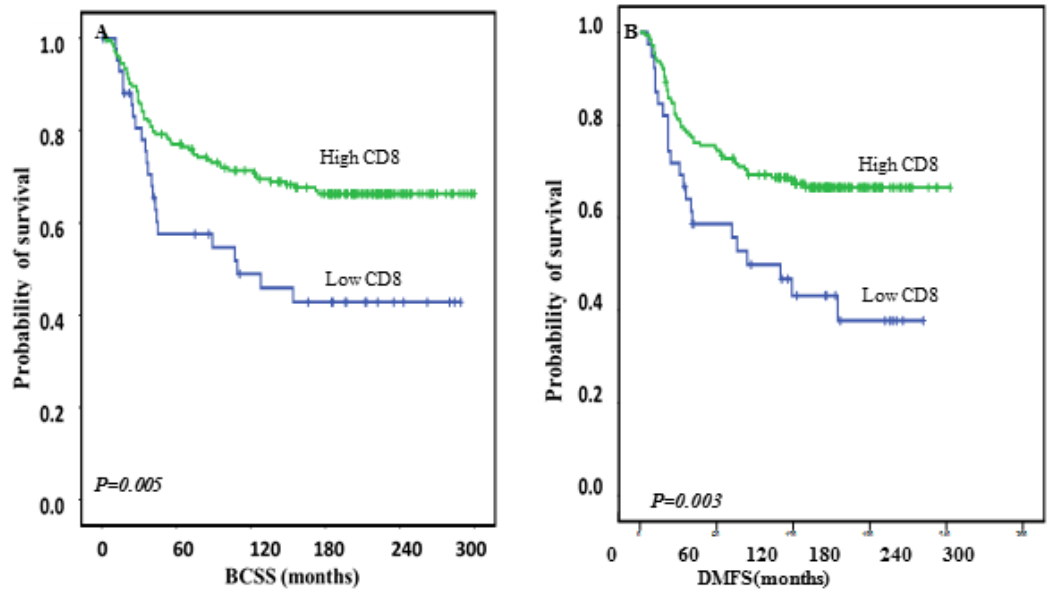
Supplementary figures



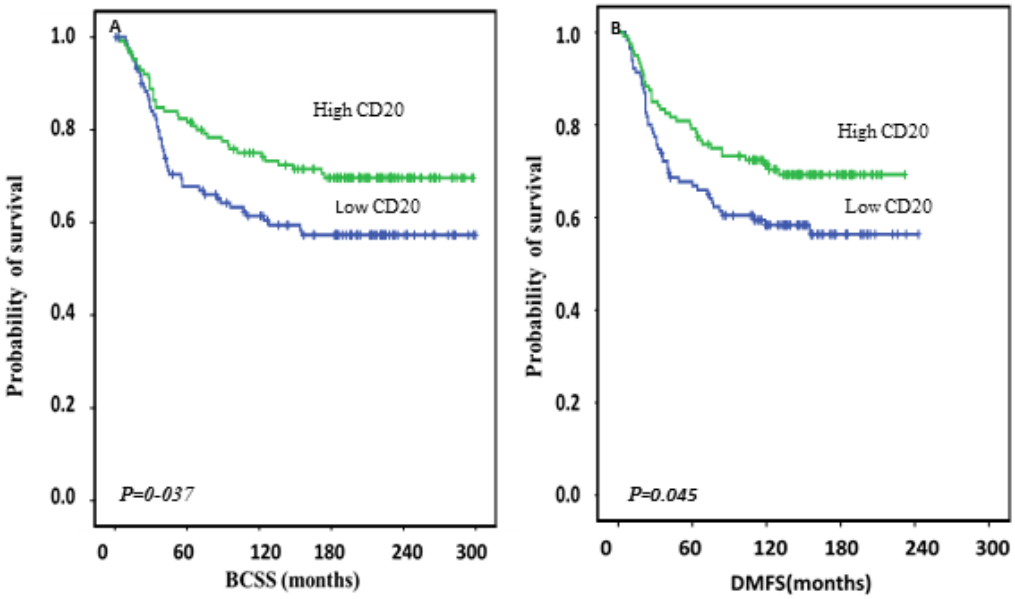
Supplementary figure 1: Kaplan Meier survival plots (A &B) showing association between FOXP3+ and CD68+ in and breast cancer specific survival (BCSS)



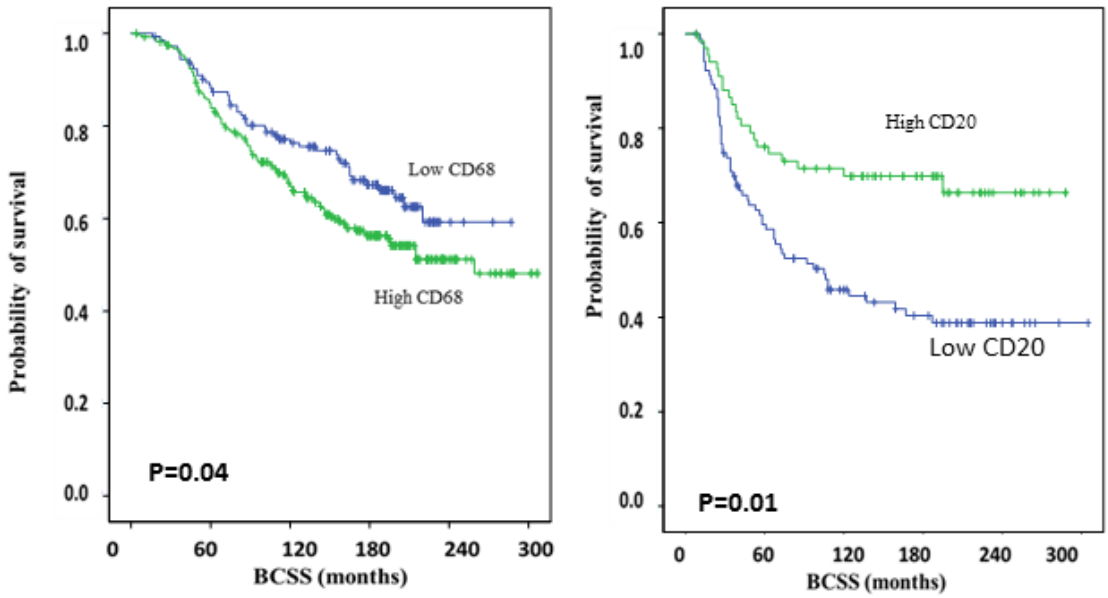
supplementary figure 2a: Kaplan Meier survival plots showing association between CD3+ lymphocytes' density in triple negative breast cancer and A) BCSS & B) Distant Metastasis Free Survival (DMFS)



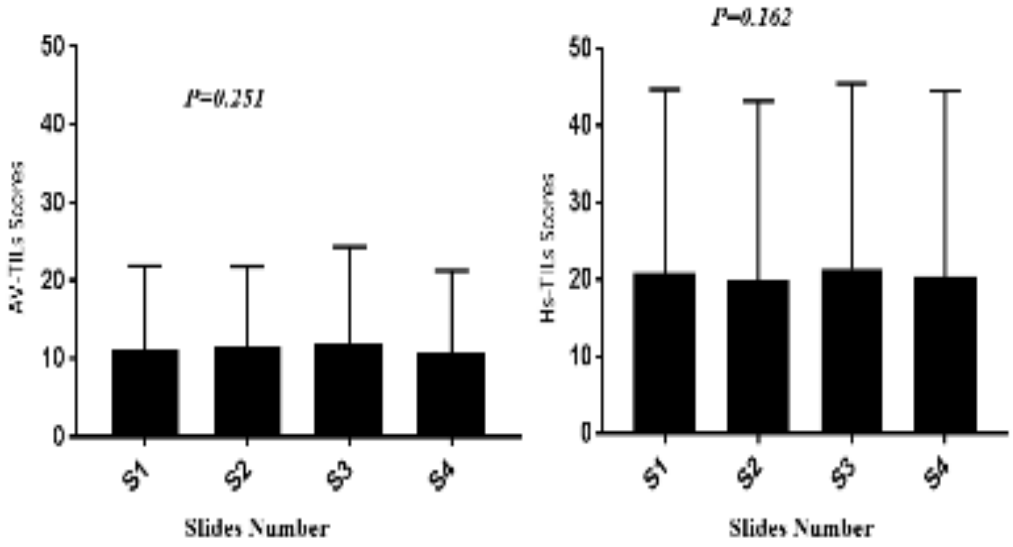
Supplementary figure 2b: Kaplan Meier survival plots for showing association between CD8+ lymphocytes' density in triple negative breast cancer and A) BCSS & B) DMFS.



Supplementary figure 2c: Kaplan Meier survival plots for showing association between CD20+ lymphocytes density in triple negative breast cancer and A) BCSS & B) DMFS.



Supplementary figure 3: A) Kaplan Meier survival plots showing association between CD68+ histiocytes' density in luminal B breast cancer subtype B) CD20+ lymphocytes' density in HER2+ enriched breast cancer and BCSS.



Supplementary figure 4 shows average TILs & hotspot TILs scores in multiple tumour blocks. S1, 2, 3 & 4 are sections 1, 2, 3, & 4 from four different tumour blocks